Table 1
Primer sequences used for RT-PCR analysis

Primer name	Forward	Reverse	Size of amplicon (bp)
CD44	CCCGAATTCATGGACAAGGT TTGGTGGCA	CCCGAATTCCTACACCCCAAT CTTCATAT	1095
GPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA	450
CD44s	TCCCACTATGACACATATTGC	ACACCTTCTCCTACTGTTGAC	548
CD44v1	TCCCACTATGACACATATTGC	TTGTGAATTACCAAACCAAG	407
CD44v2	TCCCACTATGACACATATTGC	TGTATGAAGATGACTCTTGG	428
CD44v3	TCCCACTATGACACATATTGC	TCATTTGGCTTCCAGCCTGT	358
CD44v4	TCCCACTATGACACATATTGC	TTGTCTGAAGTAGTACTTCTG	424
CD44v5	TCCCACTATGACACATATTGC	ATGTGGGGTCTCCTCTTCAT	418
CD44v6	TCCCACTATGACACATATTGC	GGAGTCTTCACTTGGGGTGG	430
CD44v7	TCCCACTATGACACATATTGC	AATCGGTCCATGAAACATCCT	396
CD44v8	TCCCACTATGACACATATTGC	GTATTTGGAGCCGCAGTAGGC	373
CD44v9	TCCCACTATGACACATATTGC	GTCTTCGCCTTCTTCCAGCTC	362
CD44v10	TCCCACTATGACACATATTGC	TTCCATTGTGTCTGGATATTG	409
Albumin	AAGGCACCCCGATTACTCCG	TGCGAAGTCACCCATCACCG	608
TAT (tyrosine aminotransferase)	TACTCAGTTCTGCTGGAGCC	GCAAAGTCTCTAGAGAGGCC	471
CK8 (cytokeratin 8)	GGAGGTGGACCCCAACATCC	CCACAGACGTGTCTGAGATC	500
Transferrin	CCTGACAAAACGGTCAAATGGTGC	TAAAAACTCTGCTGCCACAGGC	251
C/EBPα	CGGGGCCGGCGCGGGCAAGG	GGGGAATTCTCACGCGCAGTT GCCCATGG	277
Cx32	ATGAACTGGACAGGTCTATA	TCAGCAGGCTGAGCATCGGT	854
HNF4	GGGGAATTCATGGACATGGC TGACTACAG	GGGGAATTCCTAGATGGCTTC CTGCTTGG	1368
CYP1A1	GATGCTGAGGACCAGGAAGACCGC	CAGGAGGCTGGACGAGAATGC	679
CYP3A1	CAGCTCTCACACTGGAAACCTGGG	TCGAGGATCTAAACAACCTGAC	689
CYP4A3	TCGAGGATCTAAACAACCTGAC	GGTTGTGATACCTTTGGGTATGG	573
c-kit	TCCGCTGCCCCCTGACAGAC	CTACATTTTCCCCATCAGTT	600
CD34	ATGCCGGTCCACAGGGGCGC	GACTCCCGAGGTAACCAATG	900
Thy1.1	ATGAACCCAGCCATCAGCGT	TGCCGCCACACTTGACCAGT	400
CK19	ATGACTTCCTATAGCTATCG	CACCTCCAGCTCGCCATTAG	340

with the modified PBS. After washing, anti-rat IgG microbeads for MACS (Miltenyi Biotec, Bergisch Gladbach, Germany) were added. Magnetic separation was done using a MidiMACS separation unit (Miltenyi Biotec) and the positive fraction was collected. This fraction was plated on dishes and cultured in modified DMEM. Some of the sorted and cultured cells were harvested and employed for analysis by RT-PCR.

3. Results

3.1. The expression of CD44 in SHs

Using a DNA microarray, the profile of gene expression was examined to clarify the difference between SHs and MHs. We selected 164 genes with much higher expression in SHs than in MHs. From these genes, we chose 16 that possessed the transmembrane sequence (Table 3) and RT-PCR analysis was performed to confirm whether the selected genes were specifically expressed in SHs. CD44 was one of the three genes (CD44, D6.1A, BRI3) restrictedly expressed in SHs (Fig. 1A). To examine the length of the mRNA of CD44, Northern blotting was carried out. Fig. 1B shows two bands of CD44 mRNA. As CD44 is known to have many variant forms, the expression of variant forms in SHs was examined by RT-PCR. As shown in Fig. 1C, both CD44s and CD44v6 were expressed in SHs. The lengths of mRNAs of CD44s and CD44v6 detected by Northern blotting corresponded to the 1384 and 1614 bp that were expected from the sequences, respectively.

Table 2
Antibodies

Antibodies	Company or producer	Dilution
Mouse anti-rat CD44	BD Biosciences PharMingen	1:1000
Rabbit anti-rat C/EBPα	Santa Cruz	1:400
Rabbit anti-rat CK19	Generous gift from Prof. Atsushi Miyajima[a]	1:1000
Mouse anti-rat Thy1.1	Serotec	1:500
Mouse anti-rat CD44v6	Generous gift from Dr Jonathan Sleeman[b]	1:100

[a] Molecular and Cellular Biology Institute, University of Tokyo, Tokyo, Japan.
[b] Institute of Genetics, Forschungszentrum Karlsruhe, Karlsruhe, Germany.

Table 3
Data from microarray

Name of the clone	Ratio[a]
Rat mRNA for D6.1A protein	6.6
Rat CD44 protein mRNA, complete cds	15.8
Rat heat stable CD24 mRNA, complete cds	24.7
Mouse brain cDNA, clone MNCb-2630, similar to Mouse G_1	12.8
Mouse mRNA for myeloid associated differentiation protein	16.3
Rat mRNA for HB2, complete cds	8.3
Mouse tetraspan TM4SF (Tspan-6) mRNA, complete cds	6.9
Mouse mRNA for adhesion protein RA175N, complete cds	5.3
Mouse mRNA for integrin alpha6 subunit	4.5
Mouse oncostatinM specific receptor mRNA, complete cds	4.7
KDEL receptor	4.3
KIAA0404	6.9
Mouse BRI3 mRNA, complete cds	4.4
Rat mRNA for E-cadherin, complete cds	10.7
Rat mRNA for caveolin	4.1
Rat mRNA for ad1-antigen	13.2

[a] Ratio: SH/MH.

Immunocytochemistry for CD44s showed that CD44s$^+$ cells, in which the cell membrane and cytoplasm were faintly stained, first appeared at day 3. The timing of the appearance corresponded to that of SH proliferation. As SHs proliferated and formed colonies, the distribution of the protein was restricted to the cell membrane and the staining became strong (Fig. 2D and F). Colonies that consisted of CD44s$^+$ cells showed a honeycomb-like appearance (Fig. 2F), but primary cultured MHs showed no CD44s positivity (Fig. 2A and B). NPCs such as liver epithelial cells (LECs) and stellate (Ito) cells around SH colonies were not stained for CD44s either (Fig. 2F, arrow). As previously reported [7,8], large and piled-up cells sometimes appeared in SH colonies and were morphologically MHs. As the cells in a colony increased in size, CD44s-positivity gradually decreased and it was hard to detect the positivity in the piled-up cells (Fig. 2G and H, asterisks). The time course of CD44s mRNA expression is shown in Fig. 3A. CD44s expression was clearly detected from day 3 and maintained for about

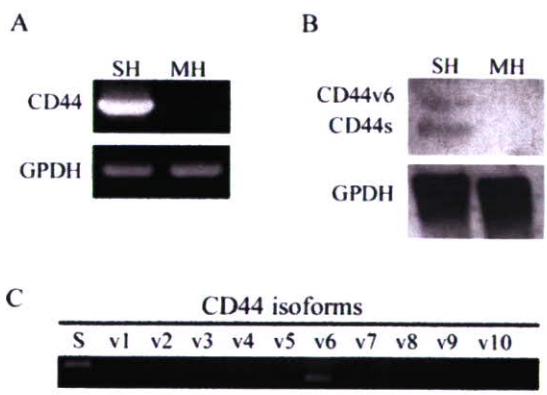

Fig. 1. Expression of CD44 in SHs cultured after cryopreservation. (A) Detection of transcript encoding CD44 by RT-PCR. Primer sets (Table I) for CD44 and GPDH were used. Total RNA was extracted from SHs that were cultured for 2 weeks after cryopreservation. Total RNA of MHs was also prepared from normal rat liver. The expected sizes of PCR products corresponding to CD44 and GPDH were 1095 and 450 basis pairs respectively. (B) Northern analysis of CD44 in SHs. 20 μg of total RNA was loaded into each lane. The filter was probed with alkaline phosphatase-linked DNA fragments specific for CD44 (upper) and GPDH (lower). Two bands of CD44 were observed in SHs. (C) Analysis of alternative splice variants of CD44 expressed in SHs. Transcripts encoding CD44 were amplified by RT-PCR using primer sets listed in Table 1. Two spliced variants (s and v6) of CD44 were expressed in SHs.

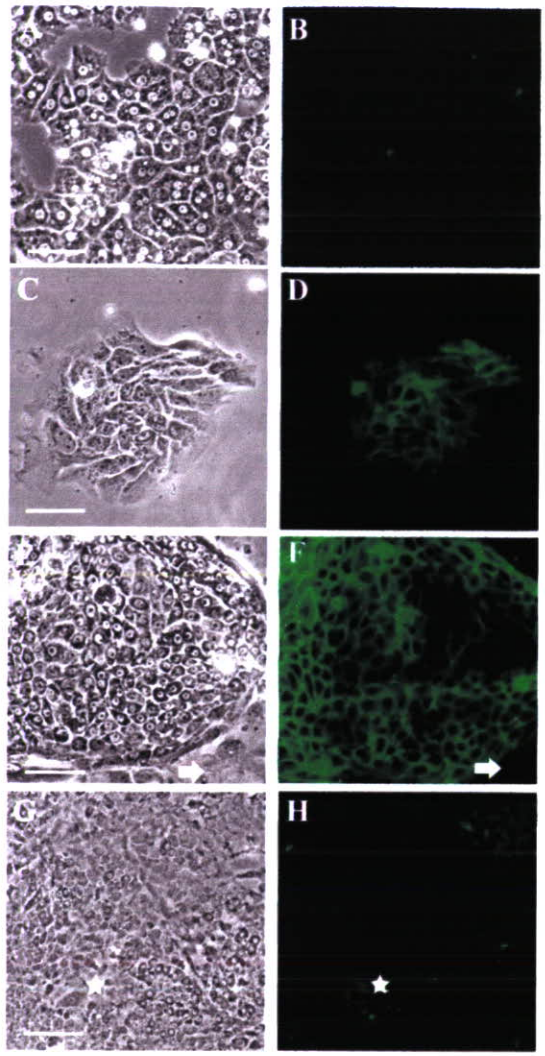

Fig. 2. Immunocytochemistry of CD44 on cultured SHs. Photographs show cell morphology under light microscopy (A, C, E, G) and immunostaining of CD44s (B, D, F, H; green). MHs isolated from a normal liver were cultured for 2 days (A and B). No expression of CD44s was observed in MHs. SHs were cultured for 5 (C and D), 18 (E and F) and 30 days (G and H). Expression of CD44 was initially observed at 3 days and reached the maximum around 18 days. CD44 disappeared from SHs by 30 days. Arrowheads indicate NPCs surrounding the SH colony (E and F). Asterisks also indicate the piled-up SHs (G and H). Expression of CD44s is faintly observed in a large, piled-up SH colony. Scale bars represent 140 μm for A, B, G, and H and 70 μm for C–F.

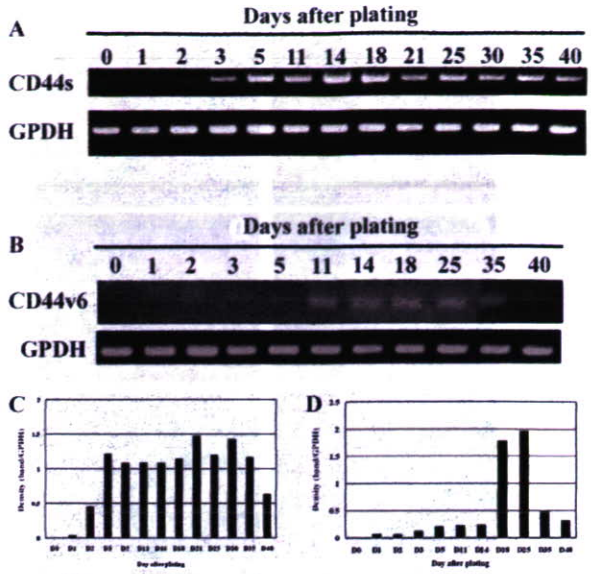

Fig. 3. Time course of CD44 expression in cultured SHs. (A) Detection of transcripts encoding CD44s by RT-PCR. SHs isolated from a normal liver were plated on culture dishes. Total RNA was extracted from cultured cells at each time point (0, 1, 2, 3, 5, 11, 14, 18, 21, 25, 30, 35, and 40 days). (B) Time course of CD44v6 expression in cultured SHs. Total RNA was extracted from cultured cells at each time point (0, 1, 2, 3, 5, 11, 14, 18, 21, 25, 30, 35, and 40 days). Primer set (Table 1) was used to detect expression of CD44v6. The expected size of the PCR product corresponding to CD44 was 430 bp. (C) Quantitation of CD44s and (D) CD44v6 expression in cultured SHs. The intensity of CD44 bands was measured by NIH image. Figure shows the density of the band normalized by GPDH. The expression of CD44s reached the maximum at around 14 days. On the other hand, maximum expression of CD44v6 was delayed compared to CD44s.

4 weeks. We also examined the expression of CD44v6 mRNA and protein in SHs. In analysis by RT-PCR, the expression of CD44v6 mRNA was detected later than that of CD44s (Fig. 3B). Immunostaining for CD44v6 showed that, in clusters of cells which were larger than SHs but smaller than binucleate MHs, the cell membranes were sometimes positive (Fig. 4).

3.2. CD44 expression with the maturation of SHs

To investigate whether the expression of CD44 was related to the maturation of SHs, Matrigel® was used to induce maturation. As shown in Fig. 5, when the cells were treated with Matrigel®, the expression of CD44s protein rapidly decreased and no expression was observed at day 4, whereas the expression of C/EBPα in MHs increased at day 4. CD44v6 expression was enhanced at day 2 and then decreased (Fig. 5). Although the expression of CD44s decreased at day 4 after the Matrigel® treatment, that of CD44v6 was not so much decreased at that time. Double staining for CD44s and C/EBPα in cultured SHs revealed that the cells, in which CD44s was strongly expressed in the cell membrane, did not possess C/EBPα $^{+}$ -nuclei (Fig. 6A). In contrast, the

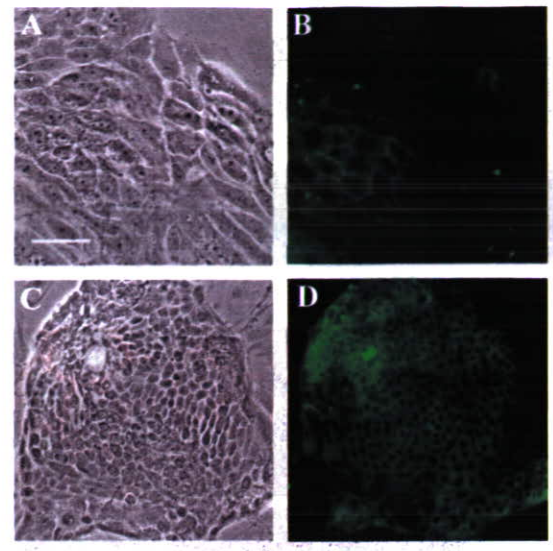

Fig. 4. Expression of spliced variant CD44v6 in cultured SHs. Photographs show cell morphology under light microscopy (A and C) and immunostaining of CD44v6 (B and D; green). SHs were cultured for 18 (A and B) and 30 days (C and D) after plating. CD44v6 appears on lateral surface of SHs.

cells in which C/EBPα protein was well expressed in the nuclei did not express CD44s in the cell membrane (Fig. 6B).

3.3. CD44 expression in vivo

Next, we examined the existence and distribution of CD44s $^{+}$ cells in the normal adult rat liver. BECs, lymphocytes, and fibroblastic cells in Glisson's capsule were positive for CD44s, whereas hepatocytes within hepatic lobules were not stained (Fig. 7A). As SHs were report-

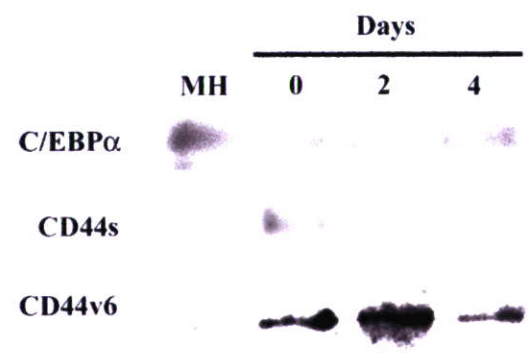

Fig. 5. Expression of C/EBPα CD44s, and CD44v6 in SHs treated with Matrigel. Immunoblotting of CD44s, CD44v6, and C/EBPα. Cell lysates were prepared from Matrigel-treated SHs and MHs. SHs were cultured for 0, 2, and 4 days after Matrigel treatment. Proteins separated by SDS-PAGE under reducing conditions were transferred onto nitrocellulose membranes followed by immunostaining with antibodies specific for C/EBPα (upper panel), CD44s (middle panel), and CD44v6 (lower panel). The expression of CD44s and CD44v6 disappeared as maturation occurred. However, the expression of CD44v6 was delayed compared to CD44s.

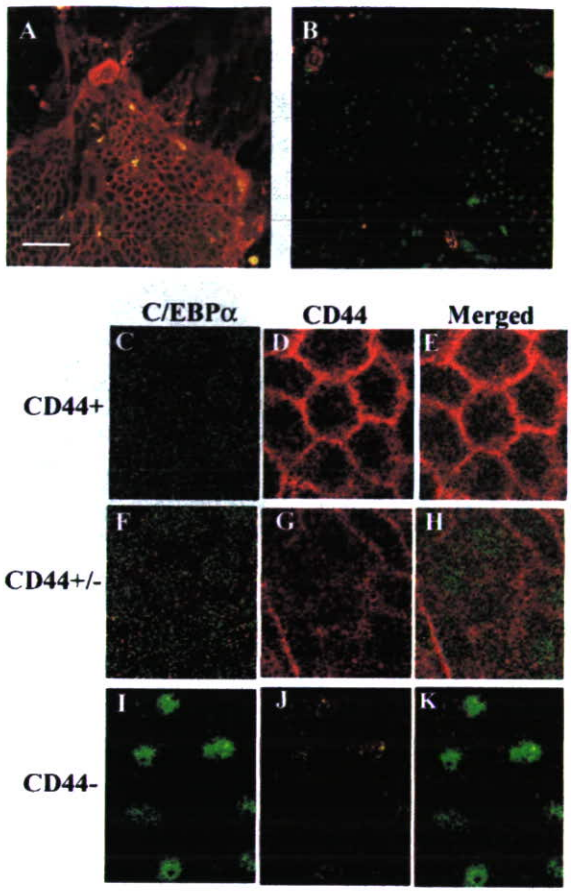

Fig. 6. Immunocytochemistry of CD44s and C/EBPα in cultured SHs. (A) Low-magnification photograph of SHs. Cells were doubly stained with a monoclonal antibody to CD44s (red) and polyclonal antibodies to C/EBPα (green). Expression of C/EBPα is only observed in matured SHs. (A) SHs were cultured for 21 and (B) 31 days after plating. Scale bars, 50 μm. (C–K) High-magnification photographs of SHs. Although CD44-positive cells did not express C/EBPα, negative cells had it in nuclei. Reciprocal expression of CD44s and C/EBPα is observed in cultured SHs.

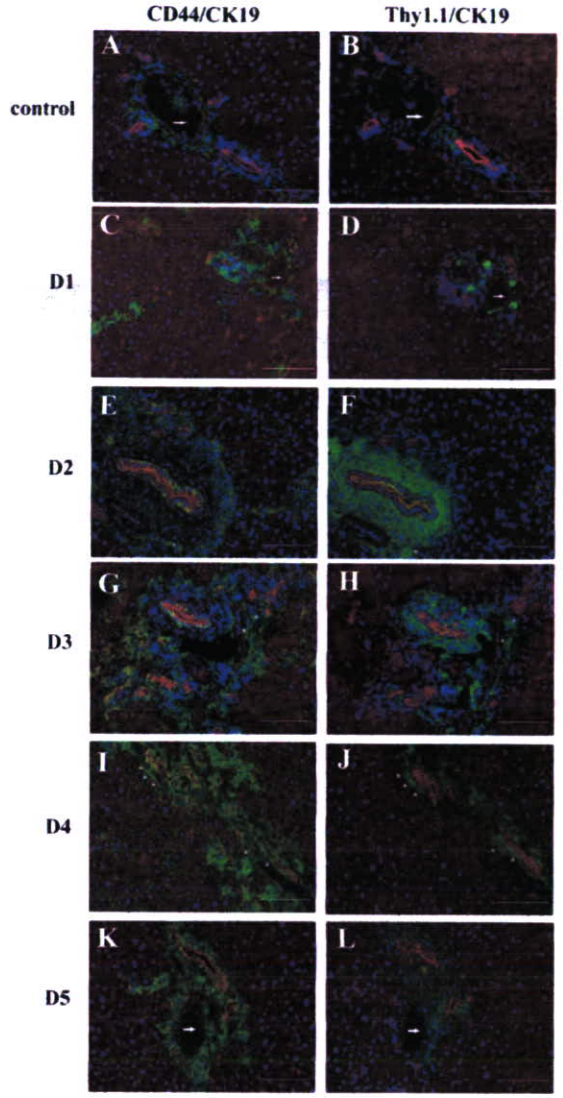

Fig. 7. Identification and localization of CD44s-positive cells in injured liver. Serial frozen sections of GalN-treated rat livers were doubly stained with antibodies to CD44s (green) and CK19 (red) (A, C, E, G, I and K) as well as Thy1.1 (green) and CK19 (red) (B, D, F, H, J and L). CK19 was used to identify BECs and Thy1.1 was used to identify oval cells. Nuclei were stained with DAPI (blue). No treatment: control rat liver; (A and B), D1; (C and D), D2; (E and F), D3; (G and H), D4; (I and J) and D5; (K and L): liver 1–5 days after treatment with GalN. Arrows show periportal region and asterisks show CD44s and Thy1.1 double-positive cells. Bile ducts are surrounded by CK-19-positive cells. Scale bars, 100 μm.

ed to appear in the rat liver severely injured by GalN [22], we examined whether those cells in the liver treated with GalN might express CD44. To further investigate the cell types that positively expressed CD44s, we carried out immunostaining using cell type-specific markers. Frozen liver sections from day 0 (control), and days 1–5 after GalN administration were double stained with antibodies to CD44s and CK19, which is a marker of BECs, and CK19 and Thy1.1, which is a marker of oval cells. Oval cells are known to be hepatic progenitor cells that appear in the GalN injury model. As shown in Fig. 7A, CD44s was expressed around the periportal area at day 0, but at days 3 and 4 CD44s$^+$ cells increased and appeared within liver lobules (Fig. 7G and I). At day 5 the number of CD44s$^+$ hepatocytes decreased compared to that at day 4 (Fig. 7K). At day 2 Thy1.1-positive cells were increased between Glisson's capsule and hepatocytes (Fig. 7F).

3.4. Sorting of CD44s$^+$ cells

As there were many CD44s$^+$/CK19$^-$ cells within liver lobules at day 4 after GalN treatment, we decided to isolate the CD44s$^+$ cells from the livers at that day. After CD44s$^+$ cells were sorted using MACS, the cells were cultured for 7 days. As shown in Table 4, about 1×10^6 CD44s$^+$ cells were separated from the GalN-treated rat, whereas few cells were separated from the normal rat. CD44$^+$ cells from the GalN-treated rat

Table 4
Recovery rate of sorted CD44$^+$ cells from GalN-treated rat liver

Treatment	Number of rats	Initial number of cells[a] ($\times 10^6$)	Number of sorted cells ($\times 10^6$)	Recovery rate (%)
Control	3	179.0 ± 1.2	> 0.01	0
GalN	5	246.0 ± 0.9	1.32 ± 0.55	0.54

[a] MHs were depleted.

could form colonies, but the cells from the normal rat could not form any colonies. Seven days after plating, most colonies formed in the dishes consisted of SHs (Fig. 8A). In Fig. 8C, the expression of mRNAs for hepatocytes, oval cells and BECs is analyzed by RT-PCR. Many hepatic markers such as albumin, transferrin, CK8, connexin32, HNF4, C/EBPα, cytochrome P450 (CYP) 1A1, and CYP4A3 were expressed in sorted CD44$^+$ cells, whereas there was no expression of markers for oval cells such as c-kit and Thy1.1, or for BECs such as CK19.

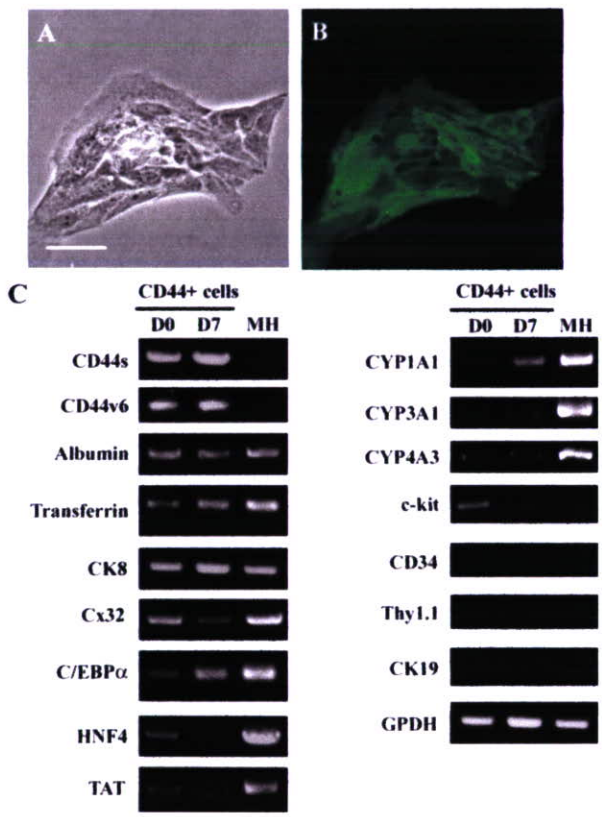

Fig. 8. Characterization of CD44-positive cells sorted from injured liver. (A) Expression of CD44s in the sorted cells. Photographs show cell morphology under light microscopy (left panel) and (B) immunostaining of CD44s in 7-day cultured cells. Scale bars, 70 μm. (C) Expression of hepatic markers in the sorted cells. Hepatic makers were detected by RT-PCR using primer sets. Total RNA was extracted from the sorted cells (day 0) and cultured CD44-positive cells (day 7). MHs were used as a positive control of hepatic maturation. Albumin, transferrin, CK8, Cx32, C/EBPα, HNF4, TAT, CYP1A1, CYP3A1, and CYP4A3 are markers for MHs; c-kit, CD34 for stem cells; Thy1.1 for oval cells, and CK19 for bile ductular cells.

4. Discussion

In the present study, we examined the gene expression profiles of SHs and MHs to find SH-specific proteins. From our analyses, we confirmed that CD44s and CD44v6 were specifically expressed in cultured SHs but not in MHs. In in vivo studies using the GalN-injury model, CD44s$^+$ hepatocytes were observed in the periportal region. The GalN-induced liver injury model was established for activation of facultative liver stem cells, i.e. oval cells. Administration of GalN causes massive necrosis of hepatic parenchymal cells in the pericentral lesion [23], which leads to the activation, proliferation, and differentiation of oval cells into hepatocytes [24–26]. As shown in Fig. 7, Thy1.1$^+$ oval cells appeared at days 2–4 in livers of GalN-treated rats. Although some Thy1.1$^+$ cells possessing relatively large cytoplasms appeared very close to Glisson's capsule at days 3 and 4, MHs with Thy1.1 expression were never observed within lobules. Furthermore, some sorted Thy1.1$^+$ cells expressed CD44s, but sorted CD44s$^+$ cells did not express Thy1.1 (data not shown). Nor could the sorted Thy1.1$^+$ cells from day 2 form any SH colonies, whereas some Thy1.1$^+$ cells from day 3 could form such colonies in our culture conditions (data not shown). These results suggested that some Thy1.1$^+$ oval cells could initially differentiate into SHs and then into MHs in regeneration of the GalN-treated liver. On the other hand, our preliminary results showed that CD44s expression occurred in the expanded SH-like progenitor cells that appeared in the retrorsine/PH-treated rat liver [27,28]. In this model, oval cells did not appear. Within the GalN-liver lobules at days 3 and 4, many CD44s$^+$/Thy1.1$^-$ cells appeared and most sorted CD44s$^+$ cells could form SH colonies in culture. Therefore, it is plausible that many CD44s$^+$ cells may come from MHs.

Expression of CD44s appeared at day 3 after plating of primary prepared SHs and increased at day 3 after GalN administration. These results suggested that CD44s expression could be up-regulated when the growth of SHs is stimulated. Therefore, although the mechanisms are at present unknown, situations where the replication of MHs is not sufficient to compensate for the loss of the cells or where SHs are free from the inhibitory signal(s) of MHs may act as a trigger for the cells, whose latent ability to be progenitors may be silenced.

A preliminary experiment provided a clue to the role of CD44 in SHs. Hyaluronic acid (HA) is a ligand for CD44. Although our data are preliminary, HA can induce the proliferation of SHs. When isolated hepatic

cells including SHs and NPCs were plated on HA-coated dishes, only SHs could proliferate to form colonies and the cells expressed CD44s. Although SECs are known to have HA receptors [29], they could not survive more than 1 week. Furthermore, the proliferating SHs on HA could not mature and the CD44s expression was maintained. In SHs, further analyses of the mechanisms by which CD44s can be induced only in SHs and how CD44 can signal the nucleus should be carried out.

The role of CD44v6 expression may be different from that of CD44s. CD44s may be related to the proliferation of SHs and to the maintenance of the capacity as progenitor cells, whereas CD44v6 may be related to their maturation because the expression of CD44v6 was delayed compared to that of CD44s in SHs and restricted to relatively large SHs. In addition, when SHs were treated with Matrigel®, the appearance of CD44v6 was delayed compared to that of CD44s. When SHs differentiated into MHs, the expression completely disappeared. CD44v6 was reported to be expressed in tumor cells, especially in metastasizing ones, and hematopoietic cells [30]. In the fetal rat liver, both CD44s and CD44v6 are expressed in hematopoietic precursor cells, including megakaryocytes [18]. In the present experiment, when SHs differentiated, the cell morphology changed from small to large and piled-up and CD44v6 transiently appeared in the cells. Therefore, the expression of CD44v6 may be related to the initiation of maturation.

In the present experiment, we clarified that CD44 was a specific marker of SHs. The expression of CD44 mRNA and protein was restricted to SHs, and was up-regulated at the time that SHs started to proliferate both in vitro and in vivo. The sorted CD44s $^{+}$ cells possessed hepatic markers, but no BEC or oval cell markers, except c-kit, were detected. Considering the present and preliminary results, we suggest that SHs are hepatic progenitor cells derived from MHs and some oval cells. Further experiments will be necessary to clarify the exact mechanisms by which their capability as progenitor cells is hidden and how the activation happens.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2006.01.029.

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Short Communication

Cytochrome P450 Expression of Cultured Rat Small Hepatocytes after Long-Term Cryopreservation

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ABSTRACT:

Small hepatocytes (SHs) are hepatic progenitor cells that can be cryopreserved for a long time. After thawing, the cells can proliferate and, when treated with Matrigel, they can differentiate into mature hepatocytes (MHs). In this study, we investigated whether cryopreserved SHs could express cytochromes P450 (P450s), whether P450 expression was induced by appropriate inducers, and whether P450 activities were measurable. 3-Methylcholanthrene (3-MC), phenobarbital (PB), pregnenolone-16α-carbonitrile (PCN), and ethanol were used as inducers for CYP1A, 2B, 3A, and 2E, respectively. Immunoblot analysis indicated that cryopreserved SHs constitutively expressed CYP1A1/2, CYP2E1, and CYP3A2 as much as 26 days after plating. Significant expression of CYP1A1/2 and 3A2 in the cells treated with Matrigel was induced by 3-MC and PCN, respectively. Although Matrigel did not up-regulate the enzymatic activity of CYP1A, CYP3A and CYP2E activities increased. Induction of CYP1A and CYP3A activities by each inducer was observed in cryopreserved cells treated with Matrigel. Although the expression of CYP2B1 could be detected in subcultured SHs treated with PB, it was not detected in cryopreserved SHs. The activity of NADPH-cytochrome P450 reductase was measured in both subcultured and cryopreserved SHs, although the activities in both were approximately 30% of that of MHs. Profiles of ^{14}C-testosterone metabolites were examined in cultured MHs and in cryopreserved SHs by high-performance liquid chromatography. Similar peaks for testosterone metabolites in MHs and SHs were observed in the same elution time. These results indicate that, although induction of CYP3A and 2B in cryopreserved SHs is inferior to that in subcultured ones, SHs can maintain the expression and activities of P450s after long-term cryopreservation.

Cytochromes P450 (P450) constitute a superfamily of monooxygenases that play a key role in either the detoxification or the metabolic activation of xenobiotics (Guengerich and MacDonald, 1990; Wrighton et al., 1992; Gonzalez and Gelboin, 1994). The P450s involved in xenobiotic metabolism are concentrated in liver cells. In vivo, many of the constitutive P450s are actually inducible by xenobiotics such as aromatic hydrocarbons, drugs, alcohol, etc. However, in traditional culture systems, it is very difficult to maintain the P450 activity of hepatocytes (Bissell and Guzelian, 1980). After cryopreservation of both rodent and human primary hepatocytes, rapid loss of hepatic differentiated functions, including P450 activity, has been reported in the cultured cells (Jackson et al., 1985; Loretz et al., 1989; Sun et al., 1990; Chesne et al., 1993; de Sousa et al., 1996; Swales et al., 1996; Garcia et al., 2003).

Small hepatocytes (SHs) have been identified as proliferating cells with hepatic characteristics (Mitaka et al., 1992, 1995; Tateno et al., 1996; Hino et al., 1999; Kon et al., 2006). We showed that a single SH could clonally proliferate and form flat colonies (Mitaka et al., 1999). It was also reported that maturation of the proliferating SHs was induced by the application of Engelbreth-Holm-Swarm sarcoma-derived matrix (Matrigel) (Sugimoto et al., 2002). Recently, SHs were shown to express several P450 proteins even after more than 1 month of culture (Miyamoto et al., 2005). In addition, the enzyme activities were induced and measured by the addition of appropriate inducers. Conversely, we reported that SHs could be cryopreserved for more than 6 months and that, even after thawing, the cells could maintain growth ability and hepatic differentiated functions (Ikeda et al., 2002).

In this study, we investigated whether cryopreserved SHs could express P450s, whether P450 expression was inducible, and whether P450 activities were measurable. The results showed that the protein expression of CYP1A1/2, CYP3A2, and CYP2E1 and the activities of CYP1A, CYP3A, CYP2E, and NADPH-cytochrome P450 reductase could be detected in the cells. Radiolabeled testosterone could be metabolized in long-term cultured cells and radioactive metabolites in cultured mature hepatocytes (MHs) were detected by high-performance liquid chromatography (HPLC).

Materials and Methods

Isolation and Culture of SHs. F344 rats (Sankyo Lab Service, Tokyo, Japan), weighing 170 to 240 g, were used. All animals received humane care, and

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ABBREVIATIONS: P450, cytochrome P450; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; EtOH, ethanol; HPLC, high-performance liquid chromatography; KH, Krebs-Henseleit; 3-MC, 3-methylcholanthrene; MH, mature hepatocyte; PB, phenobarbital; PCN, pregnenolone-16α-carbonitrile; SH, small hepatocyte.

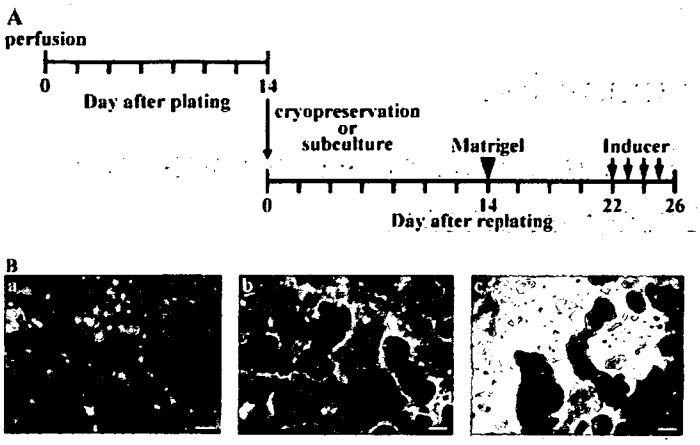


FIG. 1. A, the experimental schedule. SHs were isolated from a rat liver and cultured in modified DMEM. SH colonies were detached from the culture dishes at 14 days after plating and immediately replated or cryopreserved at −80°C for more than 1 month. One day after replating, the medium was replaced with serum-free DMEM containing 1% DMSO. Fourteen days later, the cells were overlaid with Matrigel. Arrows indicate the timing of the P450 inducer treatment. Cells at 26 days were harvested from dishes and examined for P450 expression and enzyme activities. B, phase-contrast micrographs of cryopreserved SHs at days 2 (a) and 21 (b). At day 7 after Matrigel treatment, most cells in colonies were piled up (c). All photos are the same magnification. Scale bar, 300 μm.

the experimental protocol was approved by the Committee of Laboratory Animals according to Sapporo Medical University guidelines. Hepatic cells were isolated by the two-step collagenase perfusion method. Details of the isolation and culture procedure for SHs were described previously (Mitaka et al., 1999). Finally, 6×10^4 viable cells/cm^2 were seeded on a 100-mm dish and cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical Co., St. Louis, MO) 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 (Wako Pure Chemical Co., Tokyo, Japan), 10 ng/ml epidermal growth factor (Collaborative Research Inc., Lexington, MA), 0.5 mg/l insulin, 10^{-7} M dexamethasone, and antibiotics. After 4 days of culture, 1% dimethyl sulfoxide (DMSO; Aldrich Chemical Co., Milwaukee, WI) was added to the medium.

Subculture and Cryopreservation of Small Hepatocyte Colonies. As previously reported (Mitaka et al., 1999; Ikeda et al., 2002), colonies consisting of 30 to 50 cells were observed at day 14 after plating. To collect SHs, the colonies were detached from dishes and immediately replated or cryopreserved at −80°C until use (Fig. 1A). SH colonies ($3–5 \times 10^3$ colonies/60-mm dish) were replated on dishes coated with rat-tail collagen. One day after replating, the medium was replaced with serum-free modified DMEM supplemented with 1% DMSO. Fourteen days after replating, some dishes were treated with Matrigel (1 mg/dish; Becton Dickinson, Bedford, MA).

Immunoblots for P450 Proteins. 3-Methylcholanthrene (3-MC; Wako Pure Chemical Co.), phenobarbital (PB; Wako Pure Chemical Co.), pregnenolone-16α-carbonitrile (PCN; Sigma Chemical Co.), and ethanol (EtOH; Katayama Chemical Co.) were used as inducers for CYP1A, 2B, 3A, and 2E, respectively. Eight days after Matrigel treatment, fresh medium containing the inducer (5 μM 3-MC, 2 mM PB, 2 μM PCN, or 100 mM EtOH) was added. To enhance the P450 expression, the medium containing each inducer was renewed every day for 3 consecutive days before harvest (Fig. 1A).

For immunoblots, the dishes were washed with PBS twice and then treated with 1 ml of lysis buffer (10 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 5 μg/ml leupeptin, 5 μg/ml pepstatin A) for 1 h at 4°C. The cells were scraped and used for protein extraction. Samples (15 μg/lane) were separated by SDS-polyacrylamide gel electrophoresis. Rabbit anti-CYP1A2, anti-CYP3A2, goat anti-CYP2B1, and anti-CYP2E1 (Daiichi Pure Chemical Co., Tokyo, Japan) antibodies were used for immunoblots. The details of the method were described previously (Miyamoto et al., 2005).

Enzyme Activity of P450s. For the measurement of P450 enzyme activity, the cells were washed with KH buffer (0.96% Krebs-Henseleit buffer powder, 2.5 mM $CaCl_2 \cdot 2H_2O$, 25 mM $NaHCO_3$, pH 7.5) twice at 37°C. After addition of 1.5 ml of KH buffer containing the substrates, 3 μM ethoxyresorufin, 125 μM testosterone, or 300 μM chlorzoxazone, the cells were incubated for 1 h at 37°C. The reaction reagents were collected and centrifuged at 12,000*g* for 10 min at 4°C. Then the supernatants were collected and dosed with 250 μl of methanol containing 10 μg/ml phenacetin to stop the reaction. Samples were kept at −80°C until use. Metabolites catalyzed by each P450 were fluorometrically measured according to the method of Burke and Mayer (1974) with some modifications (Miyamoto et al., 2005). P450 activities were determined by 7-ethoxyresorufin *O*-deethylation (CYP1A), testosterone-6β-hydroxylation (CYP3A), and chlorzoxazone 6-hydroxylation (CYP2E).

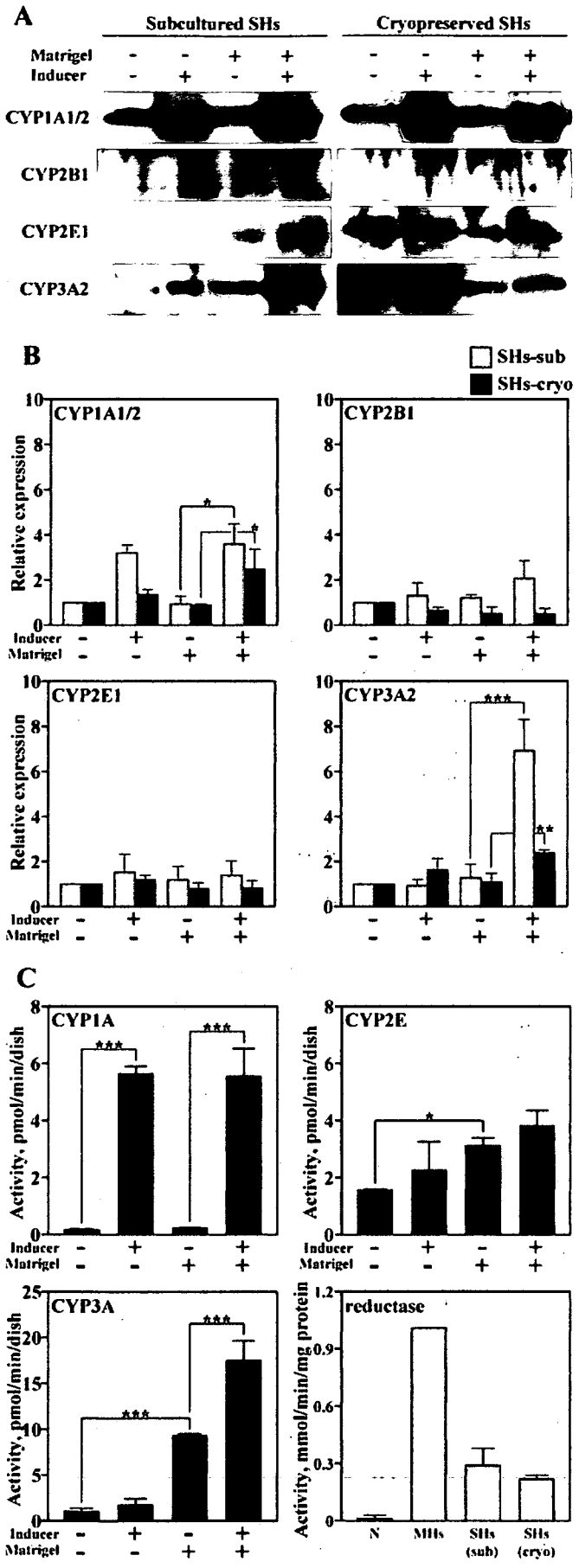
NADPH-Cytochrome P450 Reductase Activity. Activity of NADPH-cytochrome P450 reductase was measured by the method of Yukawa et al. (1983).

Profiles of ^{14}C-Testosterone Metabolites. SHs treated with Matrigel at day 22 and cultured MHs at day 1 were examined. MHs were seeded on a 100-mm dish and cultured in DMEM containing 10% fetal bovine serum. The cells were washed with KH buffer twice at 37°C. After addition of 1.5 ml of KH buffer containing 125 μM ^{14}C-testosterone (Amersham Biosciences Inc., Piscataway, NJ) to the dish, the cells were incubated for 1 h at 37°C. The reaction reagents were filtered by centrifugation (5000*g*, 5 min, 4°C) with an UltraFree-CL filter (0.45 μm; Millipore, Billerica, MA), and 100 μl was analyzed. Metabolites were detected using HPLC (LC-10ADvp; Shimadzu, Kyoto, Japan) with a Cosmosil 5C18-AR column (4.6 × 250 mm; Nacalai Tesque, Kyoto, Japan) and a radioactivity detector (FLO-ONE 525TR; Packard Instruments, Meriden, CT). The column temperature was set at 40°C and the UV detector was set at 240 nm. The mobile phase was water/tetrahydrofuran (5:1 v/v) as solvent A and methanol as solvent B. Gradient conditions were 0 to 20 min, 20 to 30% B (linear gradient); 20 to 20.5 min, 30 to 70% B (linear gradient); 20.5 to 24 min, 70% B; 24 to 25 min, 70 to 20% B (linear gradient); 25 to 30 min, 20% B. The flow rate was 1 ml/min.

Statistical Analysis. Statistical analysis was performed using Tukey's honestly significant difference test. A p value of <0.05 was considered significant.

Results and Discussion

SHs began dividing from day 3 and rapidly proliferated to form colonies. At day 14 after plating, many colonies consisting of 30 to 50 cells were detached from dishes. The colonies were replated on new dishes or cryopreserved for more than 1 month. Most subcultured colonies could attach, but only about 60% of thawed colonies could attach to collagen-coated dishes (Ikeda et al., 2002). Attached cells proliferated to form a large monolayer colony (Fig. 1B-b). Other types of cells, such as liver epithelial cells and stellate cells, also survived cryopreservation but were few in number. As reported previously (Sugimoto et al., 2002), when SH colonies were treated with Matrigel, the shape of proliferating SHs changed from flat to rising/piled-up and size from small to large. The alteration resulted in the differentiation of SHs into MHs. The cells could express not only tryptophan 2,3-dioxygenase and serine dehydratase but also liver-enriched transcription factors such as hepatocyte nuclear factors 4α and 6, and CCAAT/enhancer binding protein α and β, which are known to be restrictedly expressed in highly differentiated hepatocytes (Sugimoto et al., 2002). Expression of P450 protein and activity was also demonstrated in the SHs treated with Matrigel (Miyamoto et al., 2005). Therefore, we first examined the effect of Matrigel treatment on the morphology of cryopreserved SHs. As shown in Fig. 1B-c, the shape of most cells in colonies changed from flat to rising/piled-up within 1 week. This result was coincident with that of subcultured SHs (Miyamoto et al., 2005). We then examined the expression of P450 proteins by immunoblotting. As shown in Fig. 2, A and B, CYP1A1/2 and CYP3A2 were constitutively expressed and not induced by Matrigel in both subcultured and cryopreserved SHs. Expression of CYP2B1 was detected in subcultured SHs treated with PB but not in cryopreserved SHs. In contrast, CYP2E1 was detected in subcultured SHs treated with Matrigel but constitutively expressed in cryopreserved ones.



When SHs were treated with 3-MC and PCN for 4 days, CYP1A1/2 and CYP3A2, respectively, were induced in the cells with Matrigel. However, this induction was not observed in the cells without Matrigel. As shown in Fig. 2B, the pattern of CYP1A1/2 and CYP3A2 induction showed similarity between subcultured (3.6-fold and 6.9-fold) and cryopreserved (2.4-fold and 2.4-fold) SHs. CYP3A1 is known to be the dominant 3A after the induction. The cross-reactivity of the antibody between 3A1 and 3A2 is not clear. Therefore, the induced 3A may be 3A1 in the present experiment. Induction of CYP2E1 by EtOH was not detected in either subcultured or cryopreserved SHs.

Next, we investigated whether cryopreserved SHs could have P450 enzymatic activities. As shown in Fig. 2C, activities of 7-ethoxyresorufin *O*-deethylation (CYP1A), testosterone-6β-hydroxylation (CYP3A), chlorzoxazone 6-hydroxylation (CYP2E), and testosterone 16β-hydroxylation (CYP2B) were measured in cryopreserved SHs. In the cells without Matrigel, the activities of CYP1A and CYP3A were quite low and that of CYP2B was not detected. Enzyme activities of CYP2E and CYP3A were induced in the cells with Matrigel (2.0-fold and 8.7-fold, respectively). When SHs were treated with inducers, induction of CYP1A activity by 3-MC was observed (32-fold without Matrigel and 23-fold with Matrigel). Induction of CYP3A activity was observed in the cells with Matrigel (1.9-fold), whereas SHs without Matrigel did not show induction of CYP3A. Despite EtOH treatment, CYP2E activity did not show any significant difference between the cells with and without Matrigel. Very low activity of CYP2B was observed only in the cells with both Matrigel and PB (data not shown). There was a close relationship between the protein expression and the enzymatic activity of each P450, although some discrepancies were found; CYP3A2 protein was not adequately induced in the cells with Matrigel, and the CYP1A activity was low, although the protein was expressed. To investigate the causes of these discrepancies, we examined the activity of NADPH-cytochrome P450 reductase in SHs. As shown in Fig. 2C, the activity was observed in both subcultured and cryopreserved SHs, although measured activity was approximately 0.3-fold that of MHs. The results indicated that the reductase activity might affect P450 activity.

Although we did not detect expression and activity of CYP2B in the cryopreserved SHs, the present experiment and a previous one (Miyamoto et al., 2005) showed that subcultured SHs could express it. To determine the reason, the expression of CYP2B1 in cryopreserved SHs was measured during culture. At the time of thawing, the cells

FIG. 2. A, immunoblots for P450 proteins induced by various P450 inducers in subcultured SHs and cryopreserved SHs treated with or without Matrigel. 3-MC, PB, EtOH, and PCN were used for CYP1A1/2, CYP2B1, CYP2E1, and CYP3A2, respectively. Samples (15 μg/lane) were separated by 10% SDS-polyacrylamide gel electrophoresis. Relative expression of each P450 was estimated from immunoblots and is presented in B. C, P450 enzymes and NADPH-cytochrome P450 reductase activity. Activities of P450 proteins were induced by various inducers in cryopreserved SHs treated with or without Matrigel. The cells were exposed to KH buffer containing the substrate, 3 μM ethoxyresorufin (CYP1A), 125 μM testosterone (CYP3A), or 300 μM chlorzoxazone (CYP2E) for 1 h at 37°C, and reaction reagent was collected. Resorufin, 6β-hydroxytestosterone, and 6-hydroxychlorzoxazone were detected as metabolites. Activities of P450s (pmol/min/dish) were calculated. Activity of NADPH-cytochrome P450 reductase was measured in MHs, subcultured SHs, and cryopreserved SHs. Protein (0.1 mg) was added to buffer (1 ml) containing 0.1 M Tris-HCl (pH 7.5), 25 μM cytochrome *c*, and 0.1 mM NADPH. Reduction of cytochrome *c* was detected with a spectrophotometer (550 nm) and activity was estimated as units/mg (mM/min/mg protein). "N" indicates no protein. Excluding reductase activity of MHs, all data are means ± S.D. from three different experiments. Asterisks indicate significant induction with the inducer or Matrigel (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

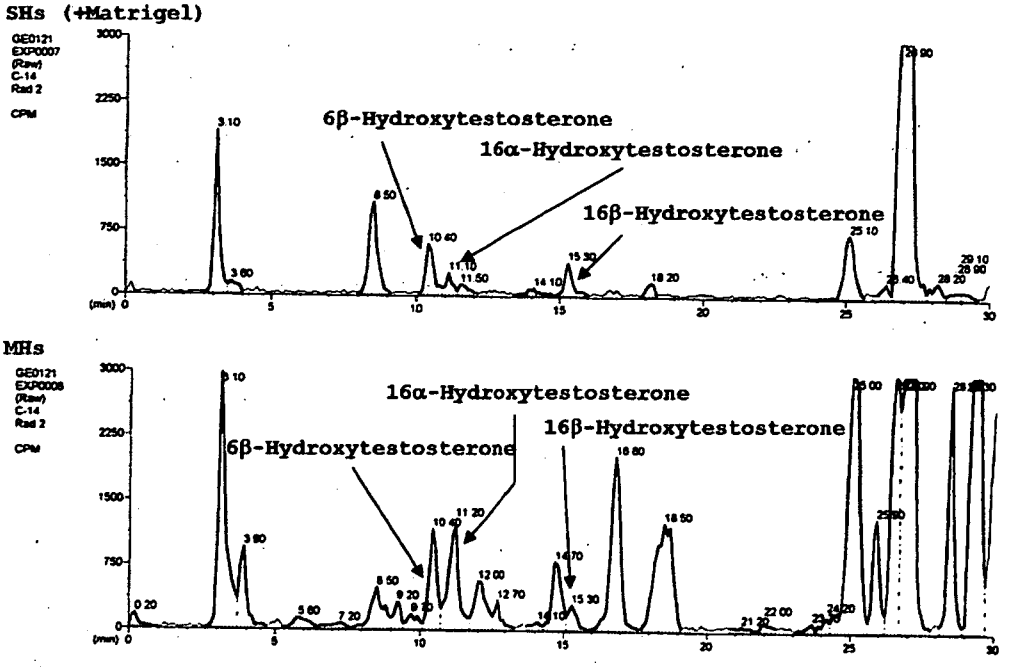


FIG. 3. Profiles of ^{14}C-testosterone metabolites in SHs and MHs. Cultured SHs at day 22 and MHs at day 1 were incubated in KH buffer containing 125 μM ^{14}C-testosterone for 1 h at 37°C. The reaction reagents were analyzed using HPLC. The main metabolites of testosterone catalyzed by P450s are indicated by arrows.

possessed the protein, but the expression rapidly decreased with time in culture. Within 3 days, the enzyme was not detected in the cells (data not shown). Further experiments are necessary to clarify the mechanism of the loss.

Next, we investigated the profiles of testosterone metabolites in SHs treated with Matrigel. As shown in Fig. 3, several metabolites of testosterone were indicated as peaks in both MHs and SHs; for example, 6β-hydroxytestosterone, 16α-hydroxytestosterone, and 16β-hydroxytestosterone. Although the heights of the peaks were relatively lower in SHs than in cultured MHs and some metabolites were not detected in SHs, testosterone could be sequentially metabolized in the cryopreserved SHs as effectively as in cultured MHs. The quite low peak of 16β-hydroxytestosterone in SHs was due to the low expression of CYP2B. The results correlated with those of both protein and enzyme activity experiments.

In the present study, we showed that cryopreserved SHs could maintain the inducibility of both P450 proteins and their enzyme activities. Until now, to supply hepatocytes used for pharmacological and pharmaceutical investigations, it has been necessary to isolate MHs for every experiment because there are few cell lines possessing hepatic differentiated functions, especially P450 enzyme activities. In addition, the number of obtainable cells depends on the number of cells in the individual because a method for proliferating hepatocytes with differentiated functions has not been established. However, by using SHs, these problems may be resolved because SHs can be isolated from adult rodents, can continue proliferating, and can be cryopreserved for a long time. Whenever hepatocytes are required, cryopreserved SHs may be thawed and plated on dishes. After the SHs proliferate and reach the required number, maturation of the cells is easily induced by Matrigel. Although improvements of the culture conditions are necessary, e.g., an increase of CYP2B expression, we think that cryopreserved SHs may be very useful for pharmacological and toxicological studies.

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Selective proliferation of rat hepatocyte progenitor cells in serum-free culture

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This protocol details a method of obtaining selectively proliferated hepatocyte progenitor cells using hyaluronic acid (HA)–coated dishes and serum-free medium. A small hepatocyte (SH) is a hepatocyte progenitor cell of adult livers and has many hepatic functions. When the rat SH begins to proliferate, CD44 is specifically expressed. To define the purification of SH, CD44 and cytokeratin 8 are used as marker proteins. The growth of SHs is faster on HA-coated dishes than on other extracellular matrix–coated ones. The use of both DMEM/F12 medium and HA-coated dishes allows the selective proliferation of SHs in culture. The purification of SHs is approximately 85% at day 10.

INTRODUCTION

Small hepatocytes (SHs) are a subpopulation of hepatocytes that have high growth potential in culture[1]. Although the cells are less than half the size of mature hepatocytes (MHs), they possess hepatic characteristics[1]. SHs can clonally proliferate to form a colony and can differentiate into MHs by interacting with hepatic non-parenchymal cells (NPCs)[2,3] or as a result of treatment with gel derived from Engelbreth-Holm-Swarm sarcoma[4]. Thus, we consider that SHs may be 'committed progenitor cells' that can further differentiate into MHs. The standard method to obtain an SH-rich fraction, which is a mixture of SHs and NPCs from a liver cell suspension after collagenase perfusion, has used multistep centrifugation. However, with this method, although SHs account for more than 60% of the cells at day 10, it is very difficult to inhibit the growth of NPCs.

Recently, gene expression analysis revealed that CD44 is specifically expressed in SHs but not in MHs[5]. The CD44 gene encodes for a family of alternatively spliced multifunctional molecules, and CD44 plays a role in adhesion of cells to an extracellular matrix such as hyaluronic acid (HA), collagen (Col) or fibronectin (FN)[6]. CD44 standard form (CD44s) is composed of a short cytoplasmic tail, a transmembrane region and two extracellular domains, and ten variant forms (v1–v10) exist. SHs have been shown to express CD44s and v6 (ref. 5). CD44s in SHs appears from 3 d after plating; expression increases with the expansion of the SH colony, but decreases with the maturation of SHs. The appearance of CD44v6 is delayed compared with that of CD44s. The expression also disappears with maturation of SHs. Although CD44 is expressed in cultured SHs, no $CD44^+$ hepatocytes are found in the normal rat liver. When the rat liver is severely injured by hepatotoxins such as galactosamine and 2-acetylaminofluorene, $CD44^+$ hepatocytes transiently appear in the periportal regions of the liver lobules. Using an anti-CD44 Ab, we have isolated $CD44^+$ cells from the galactosamine-treated rat liver[5]. These $CD44^+$ cells possess the characteristics of SHs. However, as we were not able to isolate $CD44^+$ SHs from either a normal adult liver or from a liver with two-thirds removed, we tested HA as a ligand for separating a population of SHs.

HA, a linear polymer of (1-β-4)-D-glucuronic acid (1-β-3)-*N*-acetyl-D-glucosamine, is a large glycosaminoglycan that can reach a molecular size of 10^7 Da. It is found in the tissue matrix and body fluids of all vertebrates and has diverse biological roles. These include acting as a vital structural component of connective tissues and playing roles in the formation of loose hydrated matrices that allow cells to divide and migrate, immune cell adhesion and activation, and intracellular signaling[7]. Such diversity results from the large number of hyaluronan-binding proteins (termed hyaladherins), which exhibit significant differences in their tissue expression, cellular localization, specificity, affinity and regulation. Three HA synthase (HAS) genes, coding for HAS-1, 2 and 3, are recognized to synthesize HA[8]. HAS is located at the inner cell membrane, where the newly synthesized HA is extruded into the extracellular space[9]. Synthesized HA is degraded locally in the tissues where it is produced or by the lymph nodes, and the reminder enters the bloodstream[10]. More than 90% of circulating HA is degraded by hepatic sinusoidal endothelial cells (SECs) through a receptor recycling pathway. Hyaladherins, LYVE-1 and stabilin-1 and 2, but not CD44, are expressed in SEC[11,12]. Furthermore, a relationship between serum HA levels and liver diseases has been reported[13]. HA may be related to the induction of SHs in the liver.

We found that SHs cultured on HA-coated dishes could selectively proliferate to form colonies and that the contamination by NPCs with this method was much less than with our previous method. Although we do not know in detail why a population of SHs can be isolated from a normal liver and selectively proliferate on HA-coated dishes, the HA–CD44 interaction may enhance the growth of SHs. In addition, the combination of DMEM/F12 medium and HA-coated dishes allows us to exclude FBS from the culture. Using this protocol, we have isolated human SHs from a normal adult liver. Taking into consideration the application of hepatic stem/progenitor cells to regenerative medicine, the use of proteins derived from an animal, particularly FBS, should be avoided in the culture of the cells. This protocol for isolating and culturing SHs may help researchers in this field to progress in their own investigations.

MATERIALS

REAGENTS

- Male F344 rats (Sankyo Lab Service, Tokyo, Japan) weighing 150–200 g
  ! CAUTION All animal experiments must comply with national and institutional regulations.
- Ascorbic acid-2 phosphate (Asc2P; Wako Pure Chemical Industries, Osaka, Japan, cat. no. 013-12061) (see REAGENT SETUP)
- BSA (30% solution;Serological Proteins, IL, cat. no. 82-046-3)
- Collagenase (Wako Pure Chemical Industries, cat. no. 034-10533; Yakult Pharmaceutical Industry, cat. no. YK-101; Sigma, St. Louis, MO, cat. no. C5138)
- Dexamethasone (Wako Pure Chemical Industries, cat. no. 041-18861) (see REAGENT SETUP)
- DMEM/nutrient mixture Ham F-12 (DMEM/F12) (Sigma, cat. no. D8900)
- Nicotinamide-supplemented medium[1] (to grow MHs)
- L15 medium[14] supplemented with a growth factor (to grow MHs)
- Epidermal growth factor (EGF; BD Biosciences, Bedford, MA, cat. no. 354001) (see REAGENT SETUP)
- EGTA (Sigma, cat. no. E-0396)
- Gentamicin solution (50 mg ml^{-1}; Sigma, cat. no. G1397)
- Culture medium stock (see REAGENT SETUP)
- HA derived from human umbilical cords (Biozyme Laboratories, cat. no. HA1NaL), bovine vitreous humor (Sigma, cat no. H7630), pig skin (Seikagaku Kogyo, cat. no. 400720), rooster comb (Sigma, cat. no. H5388) and *Streptococcus* (Sigma-Aldrich, cat. no. 53747) (see REAGENT SETUP)
- HANKS' balanced salt solution (HANKS; Sigma, cat. no. H9269)
- 10× Ca^{2+}, Mg^{2+}-free HANKS (Sigma, cat. no. H4641)
- Wash solution (see REAGENT SETUP)
- MH wash solution (see REAGENT SETUP)
- Phenol red–free HANKS
- HEPES (Dojindo, Kumamoto, Japan, cat. no. 342-01375)
- Insulin (Sigma, cat. no. I-5500) (see REAGENT SETUP)
- Insulin–transferrin–selenium (ITS; GIBCO, cat. no. 0459)
- $NaHCO_3$ (Kanto Chemical, cat. no. 37116-00)
- Nembutal, 50 mg ml^{-1} (Dainippon Pharmaceutical, Tokyo, Japan, cat. no. 132141)
- Nicotinamide (Sigma, cat. no. N3376) (see REAGENT SETUP)
- Penicillin–streptomycin solution (Sigma, cat. no. P-4333)
- Percoll PLUS (GE Healthcare Bio-Sciences, Piscataway, NJ, cat. no. 17-5445-01) (see REAGENT SETUP)
- L-Proline (Sigma, cat no. P5607)
- Trypan blue (Chroma Technology, VT, cat. no. 1B187) (see REAGENT SETUP)
- Pre-perfusion solution (see REAGENT SETUP)

EQUIPMENT

- Dishes, 100, 60 and 35 mm (Corning Glass Works, Corning, NY)
- Non-charged (NC) 60-mm dish (Kord-Valmark, Ontario, Canada, cat. no. 2901)
- Autoclaved 250-μm nylon filter net (Nippon Rikagaku Kikai, Tokyo, Japan)
- Cell strainer, 70- μm filter (BD Falcon, cat. no. REF352350)
- 0.2- μm filter (Mediakap-2; Spectrum Laboratories, CA, cat. no. MEM2M-02B-12S)
- Paper filter no. 3 (Advantec, Tokyo, Japan)
- Sterilized 10-cm Petri dish (glass or plastic)
- Water bath (Teitec Co., Tokyo, Japan, cat. no. EX-B2015250)
- Vascular clamp, bulldog type (Fine Science Tools, Foster City, CA, cat. no. 18050-35)
- Peristatic pump (Tokyo Rika Instruments, Tokyo, Japan, RP-1000)
- Silicon tube (ϕ4.76 × 7.94 mm^2)
- Butterfly needle, 18 gauge (Terumo, Osaka, Japan, cat. no. SV-18CLK)
- O_2 gas (95% O_2/5% CO_2)
- Neubauer improved hemocytometer (Sigma, cat. no. Z359629)

REAGENT SETUP

HA solution Measure flakes or powder of HA; UV irradiate HA on a plastic tray for 1 h. After irradiation, place HA into a 50-ml tube and adjust the concentration of HA stock solution to 10 mg ml^{-1} by adding sterilized PBS. For UV irradiation, use a standard clean bench or safety cabinet equipped with a UV lamp. ! CAUTION UV is harmful to skin and eyes.

1,000× insulin stock solution (500 μg ml^{-1}) Add 100 mg insulin to 100 ml ddH_2O and then add 1.2 ml 1 N HCl. Adjust to 200 ml with ddH_2O and filter with a 0.2-μm filter. ▲ CRITICAL Insulin dissolves in acidic solution.

Pre-perfusion solution Add approximately 850 ml ddH_2O into a 1,000-ml graduated cylinder; add 100 ml 10× Ca^{2+}, Mg^{2+}-free HANKS, 190 mg EGTA, 1 ml 1,000× insulin stock solution and stir and adjust pH to 7.5 with 7 ml 1 M $NaHCO_3$. Adjust to 1,000 ml and filter with a 0.2-μm filter. Distribute into each bottle (150 ml). Store at 4 °C until use. ▲ CRITICAL EGTA solution should be warmed to 37 °C before use.

Perfusion solution To 200 ml HANKS, add 1 ml 1,000× insulin stock solution and collagenase (100 U ml^{-1}). Shake gently. ▲ CRITICAL Prepare HANKS with insulin before the experiment. Add collagenase to pre-warmed perfusion solution just before use and immediately dissolve by gentle shaking.

Wash solution To 500 ml HANKS, add 0.5 ml 1,000× insulin stock solution, 2 ml penicillin–streptomycin solution and 0.5 ml gentamicin solution.

MH wash solution To 500 ml HANKS, add 0.5 ml 1,000× insulin stock solution, 3.3 ml BSA, 2 ml penicillin–streptomycin solution and 0.5 ml gentamicin solution.

Percoll solution[15] Add 2.4 ml 10× HANKS and 21.6 ml Percoll into a 50-ml conical tube. Mix gently upside down several times. Store at 4 °C until use.

Trypan blue stock solution (0.1%) Add 100 mg trypan blue to 100 ml phenol red–free HANKS. Filter with a paper filter.

Dexamethasone stock solution Add 39.2 mg dexamethasone to an autoclaved brown bottle. Add 10 ml ethanol (10^{-2} M stock solution); dilute to 100× (10^{-4} M) with autoclaved ddH_2O. Store at 4 °C until use. ▲ CRITICAL 10^{-4} M stock solution must be used within 3 months.

EGF stock solution (10 μg ml^{-1}) Add 10 ml autoclaved ddH_2O to an EGF vial. Store 1-ml aliquots in microcentrifuge tubes at −20 °C until use.

Nicotinamide stock solution (1M) Add 12.21 g nicotinamide to 100 ml PBS. Filter with a 0.2-μm filter and store at 4 °C until use.

Asc2P stock solution (100 mM) Add 2.90 g Asc2P to 100 ml PBS, filter with a 0.22-μm filter and store at 4 °C in a 100-ml brown bottle until use.

Culture medium stock Add the following reagents to a 1,000-ml beaker: DMEM/F12 (15.56 g), HEPES (1.20 g), L-Proline (30 mg), penicillin–streptomycin (8 ml), ddH_2O up to 1,000 ml. Mix using a magnetic stir bar, add 2.20 g $NaHCO_3$, adjust pH to 7.6 with 1 N NaOH and filter with a 0.2-μm filter. Store at 4 °C until use.

Preparation of culture medium Mix DMEM/F12 stock medium (500 ml), BSA (1.67 ml), nicotinamide stock solution (5.50 ml), Asc2P stock solution (5 ml), ITS (5 ml), EGF stock solution (0.5 ml), dexamethasone stock solution (0.5 ml) and gentamicin (0.5 ml).

EQUIPMENT SETUP

Perfusion system See **Figure 1a**. The system is composed of a water bath, vascular clamp, peristatic pump, silicon tube (ϕ4.76 × 7.94 mm^2) and 18-gauge butterfly needle.

PROCEDURE

Preparation of HA-coated dishes ● TIMING At least 1 d before the experiment

1| Dilute the stock HA solution to 100 μg cm^{-2} with PBS, fill Petri dishes and incubate at 37 °C overnight. The next day, discard the solution and wash once with PBS. Aspirate PBS and leave to dry on a clean bench with UV irradiation for 30 min.
! CAUTION UV is harmful to skin and eyes.
? TROUBLESHOOTING

Isolation of liver cells ● TIMING 1–1.5 h

2| Settle the perfusion apparatus in the warmed water bath (**Fig. 1a**). Pour the pre-perfusion solution into the apparatus before the experiment and bubble it with 95% O_2/5% CO_2 gas at a flow rate of 0.5 l min^{-1}.

▲ CRITICAL STEP The equipment should not be left for more than 30 min before proceeding to the next step.
? TROUBLESHOOTING

3| After light anesthesia by ether, anesthetize a rat with an i.p. injection of nembutal (5 mg per 0.1 ml per 100 g body weight).
? TROUBLESHOOTING

4| Cut the abdominal wall using surgical scissors and open the abdominal cavity to look at the portal vein.

5| Ligate a common bile duct and splenic vein together using a surgical thread at the portion nearest the portal vein (**Fig. 1b**).
▲ CRITICAL STEP This is to avoid loss of the solution.
? TROUBLESHOOTING

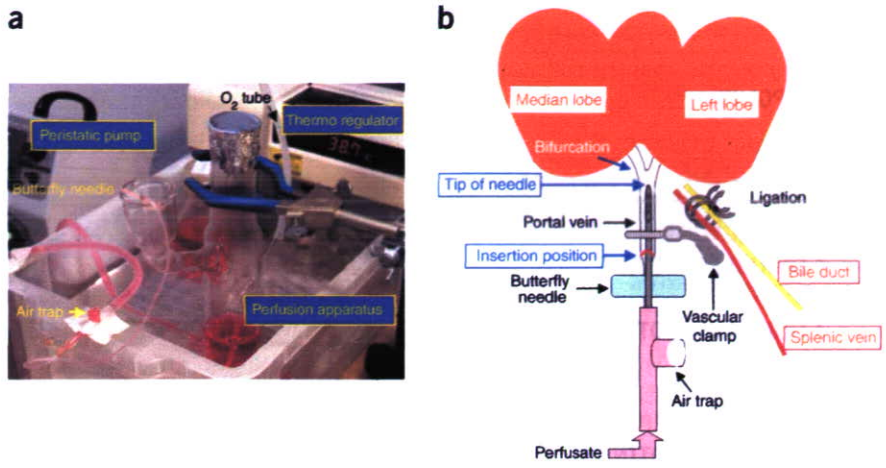

Figure 1 | Experimental setup. (**a**) Setting up the perfusion apparatus. Pre-perfusion solution is warmed to approximately 39 °C and bubbled with O_2. (**b**) Position of the inserted needle in the portal vein of a rat.

6| Insert a butterfly needle filled with pre-perfusion solution into the portal vein 1.5–2.0 cm from the bifurcation of the portal vein, stop the tip of the needle at a position close to the bifurcation of the portal vein and clamp the needle with a surgical clip (**Fig. 1b**).
? TROUBLESHOOTING

7| Start the perfusion at a flow rate of 30 ml min^{-1}.

8| Cut the inferior vena cava and heart as soon as the flow starts.
▲ CRITICAL STEP Cut the inferior vena cava at the portion beneath the right kidney and the thoracic cavity, and then cut the heart to flow the perfusate out of the cadaver. Washing out the blood completely from the liver and preventing an increase of intra-hepatic pressure is important to the success of the preparation.
? TROUBLESHOOTING

9| When the amount of the pre-perfusion solution becomes small, add collagenase to the perfusion solution and then pour it into the perfusion apparatus.
▲ CRITICAL STEP To avoid a decrease in collagenase activity, dissolve it after the pre-perfusion solution flows. Do not mix vigorously.
? TROUBLESHOOTING

10| Flow the solution at a flow rate 15–20 ml min^{-1}.
▲ CRITICAL STEP The flow rate should be decided by rat body weight.
? TROUBLESHOOTING

11| Stop the flow before air bubbles move into the liver when the solution flows out from the reservoir.
? TROUBLESHOOTING

12| Cut the liver from the abdominal cavity and transfer it to a sterilized Petri dish.

13| Prepare a 100-ml beaker with 70–80 ml wash solution and add a small amount of the wash solution to the Petri dish.
▲ CRITICAL STEP From this step onward, all procedures should be done in sterilized conditions.

14| Peel the hepatic capsule as carefully as possible and, to drop the digested cells, shake the liver into the beaker.
? TROUBLESHOOTING

15| Filter the cell suspension through a 250-μm nylon filter net into a new 100-ml beaker.

16| Filter the cell suspension through a 70-μm filter into four 50-ml conical tubes using a 25-ml pipette and then adjust each tube to an equal volume with the wash solution (approximately 40 ml).

17| Centrifuge the tubes at 50*g* for 1 min at 4 °C.
? TROUBLESHOOTING

18| Collect supernatants and transfer to new conical tubes. Repeat Steps 15–17 a total of 3 times.
▲ CRITICAL STEP Many SHs are included in the supernatant after low-speed centrifugation. This procedure is carried out to remove the majority of MHs from the cell suspension.
■ **PAUSE POINT** Cells in tubes may be kept on ice for 1–3 h. Avoid long-term preservation.

19| If you want to isolate MHs, proceed directly to Step 19B. To isolate SHs, proceed with Step 19A.

(A) Isolation of SHs ● TIMING **1–1.5 h**

(i) Centrifuge the supernatant at 50*g* for 5 min at 4 °C.
▲ CRITICAL STEP This procedure is used to remove hematopoietic cells.
? TROUBLESHOOTING

(ii) Discard the supernatant and add 40 ml wash solution to the tubes.
? TROUBLESHOOTING

(iii) Centrifuge at 50*g* for 5 min at 4 °C after gentle pipetting to dissociate the cell pellet.

(iv) Add 40 ml wash solution and then centrifuge at 150*g* for 5 min at 4 °C.
▲ CRITICAL STEP This procedure is carried out to damage some MHs contained in the suspension.
? TROUBLESHOOTING

(v) Discard the pellet, add 40 ml wash solution and then centrifuge at 150*g* for 5 min at 4 °C.

(vi) Discard the supernatant, pour 20 ml culture medium into each tube and gather the suspension into two 50-ml conical tubes.

(vii) Centrifuge the suspension at 50*g* for 5 min at 4 °C.
? TROUBLESHOOTING

(viii) Add 10 ml culture medium to each tube and gather the suspension into one tube.

(ix) Add 0.5 ml cell suspension to 1.5 ml trypan blue solution and pipette gently.
▲ CRITICAL STEP Tubes should be on ice.

(x) Count the viable cells as soon as trypan blue is added. For counting, use a Neubauer improved hemocytometer. Count the number of cells with trypan blue–negative nuclei which exist in nine masses. Use this formula to obtain the cell number: the number of viable cells (cells ml^{-1}) = [(number of cells inside nine masses)/9] × 4 × 10^4.
▲ CRITICAL STEP Cells with trypan blue–positive nuclei are dead. Count the number of cells that are smaller than typical MHs and larger than NPCs (15–20 μm in diameter). The overall viability of cells will not be good, but most small cells, including SHs, are viable. As SH colony formation depends on the cell density, the number of viable cells, which have the potential to attach to the dishes, is important in this step.
■ **PAUSE POINT** Cells in tubes may be kept on ice for 1–3 h. Avoid long-term preservation.

(xi) Adjust the concentration of the cells to 1 × 10^5 cells ml^{-1} and then seed them on HA-coated dishes.
? TROUBLESHOOTING

(xii) Place dishes in a 5% CO_2/95% air incubator at 37 °C.

(xiii) Replace the medium with fresh medium after 3 h.
▲ CRITICAL STEP This procedure excludes unattached cells.

(xiv) Renew medium every other day.
▲ CRITICAL STEP Two times a week is enough when the number of attached cells is small.

(B) Isolation of MHs ● TIMING **1–1.5 h**

(i) Add 20 ml MH wash solution to a pellet in each tube (from Step 17) and gather the suspension into two 50-ml conical tubes.

(ii) Centrifuge the tubes at 50*g* for 1 min at 4 °C after gentle pipetting to dissociate the cell pellet.
▲ CRITICAL STEP As MHs are more labile to a shock-like pipetting or centrifugation than SHs, gentle pipetting is important.
? TROUBLESHOOTING

(iii) Discard the supernatant and add 40 ml MH wash solution to each tube.

(iv) Centrifuge at 50*g* for 1 min at 4 °C.
? TROUBLESHOOTING

(v) Discard the supernatant and add 25 ml MH wash solution to each tube.

(vi) Pipette gently to dissociate the cell pellet.

(vii) Pour 25 ml cell suspension into Percoll solution and gently mix (upside down several times).

(viii) Centrifuge at 50*g* for 15 min at 4 °C.
▲ CRITICAL STEP This step is performed to remove dead MHs. After centrifugation, dead MHs and NPCs float on the solution.
? TROUBLESHOOTING

(ix) Discard the supernatant and add 40 ml MH wash solution to each tube.

(x) Centrifuge the tubes at 50*g* for 1 min at 4 °C after gentle pipetting to dissociate the cell pellet.
? TROUBLESHOOTING

(xi) Discard the supernatant and add 40 ml culture medium to each tube.
(xii) Centrifuge the tubes at 50*g* for 1 min at 4 °C after gentle pipetting to dissociate the cell pellet.
? TROUBLESHOOTING
(xiii) Add 20 ml culture medium to each pellet and gather the suspension into one tube.
(xiv) Add 0.5 ml cell suspension to 1.5 ml trypan blue solution and suspend.
(xv) Count the number of viable and dead MHs as described in Step 19A(x).
▲ CRITICAL STEP Keep cells in tubes on ice.
(xvi) Adjust cell density to 2–6 × 10^5 viable cells ml^{-1} and then seed the cells on dishes.
? TROUBLESHOOTING
(xvii) Place the dishes in a 5% CO_2/95% air-incubator at 37 °C.
(xviii) 1–2 h later, replace the medium with fresh medium.
▲ CRITICAL STEP If researchers need to grow MHs, we suggest using nicotinamide-supplemented medium[1] or L15 medium[14] supplemented with a growth factor.
? TROUBLESHOOTING

● TIMING
Steps 2–18, isolation of liver cells: 1–1.5 h
Steps 19A(i–xiv), isolation of SHs: 1–1.5 h
Steps 19B(i–xviii), isolation of MHs: 1–1.5 h

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
Reagent setup	Reagent bacterial or viral contamination or lost activity	Inadequate sterilization	To sterilize hyaluronic acid (HA) solution, avoid using an autoclave or filter. Instead, use UV-irradiation and HA will then dissolve in sterilized PBS
		Storage conditions	Stock solution is kept at 4 °C. To avoid bacterial contamination, a small amount of the stock solution should be prepared for several experiments. Avoid long-term storage (more than 1 mo)
Reagent setup	HA insolubility	Short time	As HA solution has high viscosity, HA is dissolved overnight at 37 °C
Reagent setup	Crystallization of nicotinamide	Concentrated	Crystals are sometimes formed in 1 M nicotinamide stock solution. As they may damage cells, do not use the solution. Make new stock solution
	Quantity of collagenase	Difference between lots and between suppliers	Adjust the quantity of collagenase by units, not percentage
Step 1	Dish coating	Unequally distributed	Shred the solution using a non-charged dish. If there is not enough HA solution to cover the dish surface, add more PBS. HA-coated dishes can be stored at 4 °C, but use within 1 mo
Step 2	O_2 gas bubbling	Changes of solution color	Too much O_2 gas flow causes the buffer to become yellowish. If the color changes, stop the O_2 gas. Viability of small hepatocytes (SHs) decreases in acidic conditions
Step 3	Animal moving	Light anesthesia	Inject a small additional amount of nembutal
Steps 5, 6, 8–11	Poor yield or viability, and cell aggregation	Collagenase	(i) Collagenase activity is best at 37 °C. To keep the perfusion-solution at 37 °C, carefully adjust the temperature of the water bath and solution at the tips of tubes (ii) Long-term storage (more than 1 y) causes loss of activity (iii) Storage should be at 4 °C
		Length of perfusion time	(i) Retention of collagenase solution in the abdominal cavity causes the early rupture of hepatic capsules and inadequate digestion

TABLE 1 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
			(ii) Although collagenase works efficiently at a slow flow rate, too slow a flow causes partial perfusion of the liver, and the yield and viability of the cells have a tendency to be bad
		Flow rate	The flow rate depends on the weight of the rat. When the weight is more than 250 g, use the maximum flow rate of 20 ml min^{-1}. Liver weight is not correlated to body weight in bigger rats. A relatively high flow rate may cause the early rupture of hepatic capsules
		Embolisms	(i) Inside of the needle is not filled with perfusate (ii) Do not shake vigorously after collagenase is added to perfusion solution (iii) Tube sometimes generates bubbles and causes air embolisms in the liver. An air-trap apparatus is necessary. A rapid change of liver color from red-brown to light brown is a sign of good circulation of the perfusate. Red spots in the liver may result from embolisms
Step 14	Shortage of cells	Insufficient extraction of cells	Comb the liver remnant carefully using tweezers at Step 12
Steps 17, 19A(i), (ii), (iv) and (vii), 19B(ii), (iv), (viii), (x) and (xii)	Recovery of cells	Time of centrifugation	Centrifugation should be adjusted to g_{max}. The times shown are from pushing the 'Start' button to pushing the 'Stop' button
Step 19A(xi)	Poor growth of SHs	Few SH colonies	(i) Avoid plating too small a number of cells. We found that fewer than 2.5×10^3 cells cm^{-2} resulted in a smaller number of SH colonies than we expected (ii) Total number of SH colonies is dependent on rats. Cells from younger rats make many colonies per dish[19]
Step 19B(viii)	No pellet	Poor mixture	Mix the cell suspension again and repeat Step 8. Good mixing of cells and Percoll solution is important to obtain high viability and purity of mature hepatocytes (MHs)
Step 19B(xvi)	Poor attachment	Too many cells	9×10^5 cells and 2×10^6 cells are confluent in 35-mm and 60-mm dishes, respectively
		Few cells	Avoid using fewer than 1×10^4 cells cm^{-2}. MHs need cell–cell contact to survive. Confluence is important to maintain hepatic differentiated functions and cell longevity
		Dish	In serum-free culture, use extracellular matrix (collagen type I or IV, fibronectin, laminin and vitronectin)–coated dishes. Type I collagen (rat tail collagen) is sufficient for good attachment of MHs. You can also use pre-incubation of serum-supplemented medium for 1 h
Step 19B(xviii)	No MH growth	Medium	(i) Avoid the combination of L15 medium and nicotinamide because many cells may die (ii) Please refer to review article[1]
		Growth factors	Epidermal growth factor, hepatocyte growth factor and transforming growth factor-α have similar growth effects on MHs. MHs require high-quality water. Water in some places may not be appropriate for the culture even if other cells can grow
		Water	Deionization may not solve this problem. We recommend using purchased water; otherwise you can obtain it from a successful laboratory

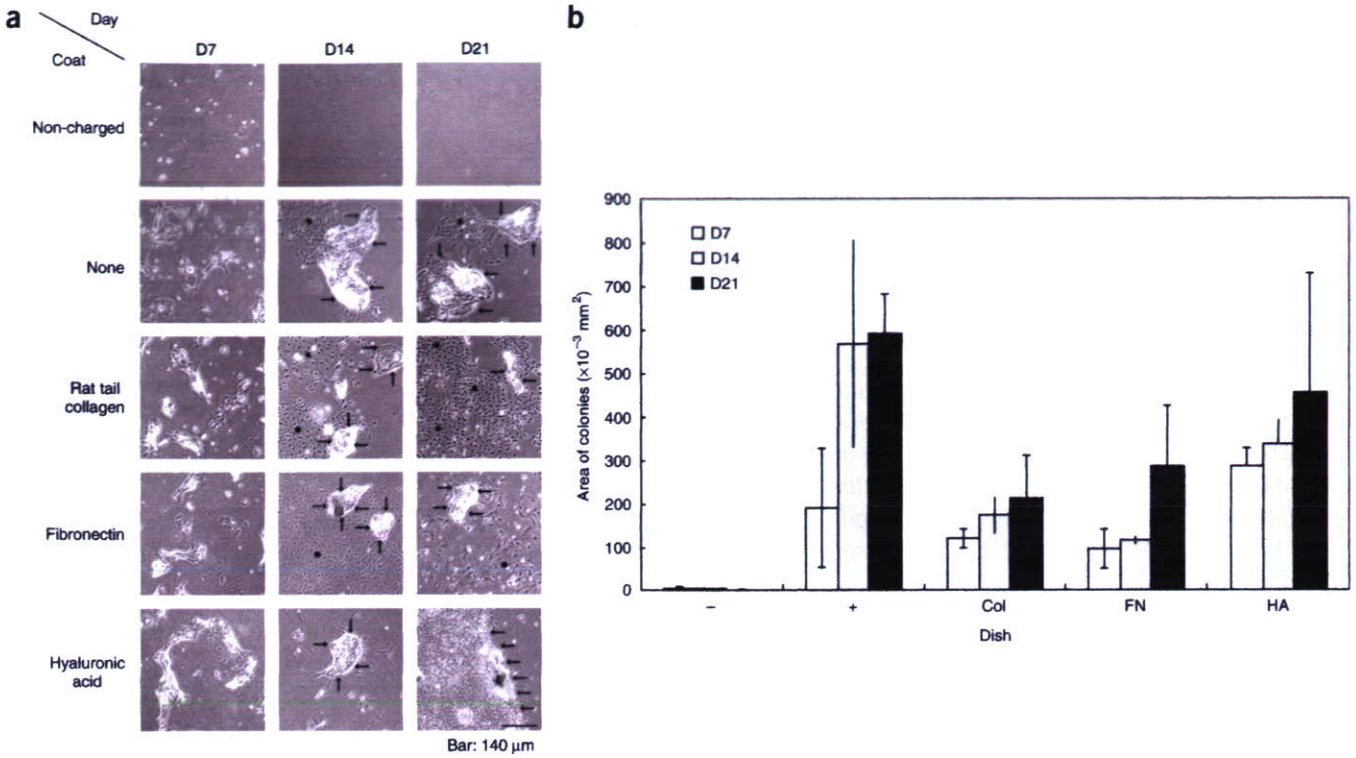

Figure 2 | Growth of liver cells on dishes coated with extracellular matrices. (**a**) Phase-contrast micrograph of small hepatocytes (SHs) and non-parenchymal cells at days 7, 14 and 21 after plating. Cells are cultured in DMEM supplemented with 10% FBS. Arrows show SH colonies and asterisks show NPCs. The expansion of SH colonies is clear and there is little contamination by NPCs on the hyaluronic acid (HA)–coated dish compared with other dishes. At day 21 on HA-coated dishes, proliferated cells, which are marked by arrows, are all SHs. The magnification of the photos is the same; scale bar = 140 μm. (**b**) The growth of SH colonies on various dishes. Columns and bars show the average and s.d. of three dishes, respectively.

TABLE 2 | Purity of small hepatocytes cultured in serum-free medium and on hyaluronic acid–coated dishes.

Medium	$CD44^+$ cells (%)	$CD44^-$ cells (%)
DMEM/F12-FBS (HA-coated dish)	84.5 ± 3.2	15.5 ± 3.2
DMEM+FBS (Normal dish)	64.1 ± 8.0	35.9 ± 8.0

The numbers show the average ± s.d. of three dishes. Cells were cultured for 10 d and immunostained for CD44. After counterstaining with hematoxylin, both $CD44^+$ and $CD44^-$ cells were counted. $CD44^-$ cells include sinusoidal endothelial, stellate, liver epithelial and Kupffer cells.

ANTICIPATED RESULTS

A critical component of this method is to use HA-coated dishes and serum-free medium for SH culture. As CD44 is not expressed in MHs but is expressed in SHs, we first examined whether SHs could selectively proliferate on HA-coated dishes under our standard culture conditions (DMEM + 10% FBS). As shown in **Figure 2a**, compared with the NC-, non-, rat tail Col- and FN-coated dishes, SHs could selectively proliferate on HA-coated dishes. However, there was much less growth of NPCs in HA-coated dishes than in other dishes. The average size of SH colonies on HA-coated dishes was larger than that of SH colonies on NC-, Col- and FN-coated dishes (**Fig. 2b**). Although the SH growth on non-coated dishes was as good as that on HA-coated dishes, NPCs also grew (**Table 2**).

Next, we examined whether SHs could proliferate in serum-free medium.

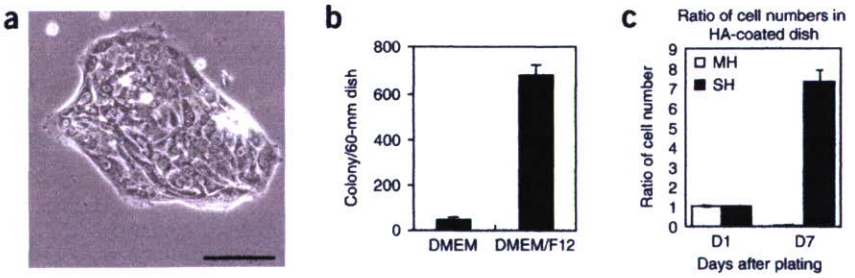

Figure 3 | The growth of small hepatocyte (SH) colonies on hyaluronic acid (HA)–coated dishes cultured in serum-free medium. (**a**) Phase-contrast micrograph of a typical SH colony at day 7. Scale bar = 100 μm. (**b**) The number of SH colonies on HA-coated dishes cultured in serum-free DMEM or DMEM/F12 medium at day 10. Many colonies grew in the serum-free DMEM/F12 medium, whereas there were few in the serum-free DMEM. Columns and bars show the average of the total number of colonies per 60-mm dish and s.d. of three dishes, respectively. (**c**) Few mature hepatocytes (MHs) can survive in the serum-free medium on HA-coated dishes. The growth of MHs and SHs is shown as the ratio day 7/day 1.

Although we reported that SH colonies could expand in serum-free medium after re-plating SH colonies[4,16], it was very difficult for SHs in serum-free medium to grow to form colonies on conventional dishes. When we used serum-free DMEM/F12 and HA-coated dishes, many colonies developed (**Fig. 3a**), whereas few colonies grew in serum-free DMEM (**Fig. 3b**). We usually use HA derived from human umbilical cords. However, the effect of HA is not different among commercially available forms of HA derived from pig skin, bovine vitreous humor, rooster comb and *Streptococcus* (data not shown). To observe the appearance of SHs in serum-free culture, both nicotinamide and growth factors (either a single factor or a combination of EGF, hepatocyte growth factor and transforming growth factor-α) must be included in the medium[1]. These results suggest that ingredients contained in Ham F12 medium are important for SHs to grow in the serum-free culture, because the use of DMEM alone without FBS cannot support the growth of SHs. However, when the specific agents included in Ham F12 medium were added to DMEM and SHs were cultured in the serum-free medium, the growth of SHs was neither enhanced nor inhibited (data not shown). Therefore, we now hypothesize that the balance of ingredients in Ham F12 medium may be important for SHs to grow in serum-free culture. On the other hand, adding transferrin and selenium is different from the original recipe for culturing rat SHs[2]. Although SHs appear and grow without those agents, both are necessary to maintain their growth in serum-free culture. Without transferrin and selenium, the growth of SHs becomes worse after 1–2 weeks of culture (current data not shown; refer to ref. 1). **Figure 3a** shows a typical SH colony in serum-free culture. Although it was difficult to avoid MH survival in DMEM with FBS, the use of HA-coated dishes and serum-free medium suppressed MH survival. Most attached MHs died within 1 week (**Fig. 3c**). In this protocol, the purity of SHs was 84.5% ± 3.2% at day 10 (**Table 2**). Some SECs ($SE1^+$), stellate cells ($desmin^+$), liver epithelial cells ($vimentin^+$) and Kupffer cells ($ED1/2^+$) were observed.

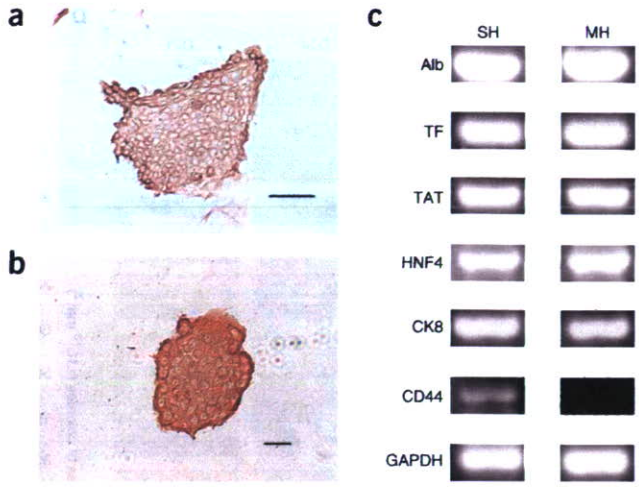

Figure 4 | Characteristics of small hepatocyte (SH) colonies on hyaluronic acid (HA)–coated dishes. Immunocytochemistry for (**a**) CD44 and (**b**) CK8 at day 10. Scale bars = 100 μm. (**c**) Expression of mRNAs of hepatic marker genes. SHs were cultured in serum-free medium on HA-coated dishes at day 7. RNA of mature hepatocytes (MHs) is derived from isolated MHs. SHs express many hepatic marker genes. CD44 is expressed in SHs but not MHs. These are representative data.

Characterization of SHs on HA was carried out using immunocytochemistry and RT-PCR. All SHs were immunocytochemically stained with anti-CD44 (**Fig. 4a**) and anti-CK8 Abs (**Fig. 4b**). As shown in **Figure 4c**, RT-PCR shows that SHs express mRNAs of hepatic marker genes such as albumin, transferrin, tyrosine aminotransferase, hepatocyte nuclear factor-4α and CK8 as plentifully as MHs at day 10. In addition, CD44 is not expressed in MHs but is expressed in SHs. These results reveal that cell colonies grown in serum-free DMEM/F12 on HA have quite similar characteristics to SHs observed using our standard culture method (DMEM/FBS). Until now, we have not observed any difference between the cells.

The SH is a hepatocyte progenitor cell that is committed to differentiate into an MH. Manipulation of the growth and maturation of SHs is easy, and they can be cryopreserved[17]. Cryopreserved SHs can maintain the abilities of growth and maturation[18]. Maturated SHs in serum-free culture can be used for studies of hepatic drug metabolism, liver diseases and regenerative medicine.

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