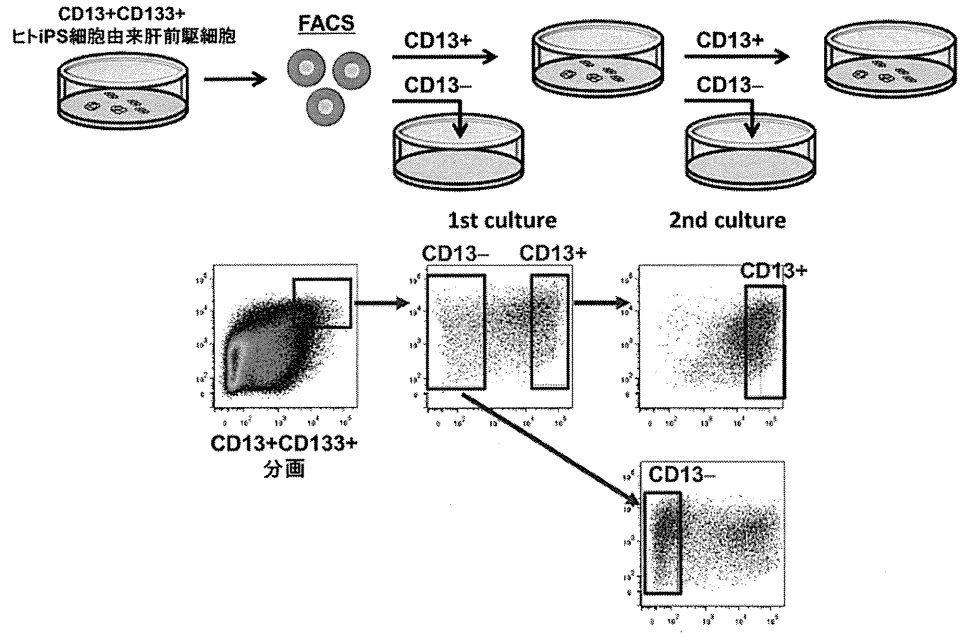


CD13+細胞： Self-renewal-likeな活性を持つ高増殖性細胞
ヒトiPS細胞由来肝前駆細胞のin vitro expansionのターゲット細胞

図7 ヒト iPS 由来肝幹・前駆細胞の長期培養における正常変化

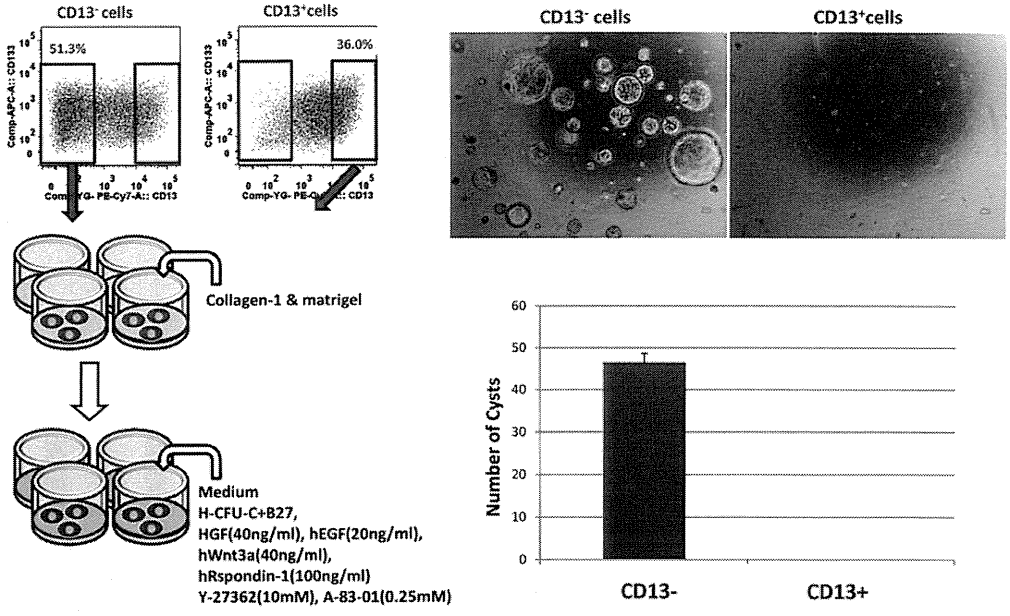


図8 ヒト iPS 由来肝幹・前駆細胞の胆管様構造への分化能

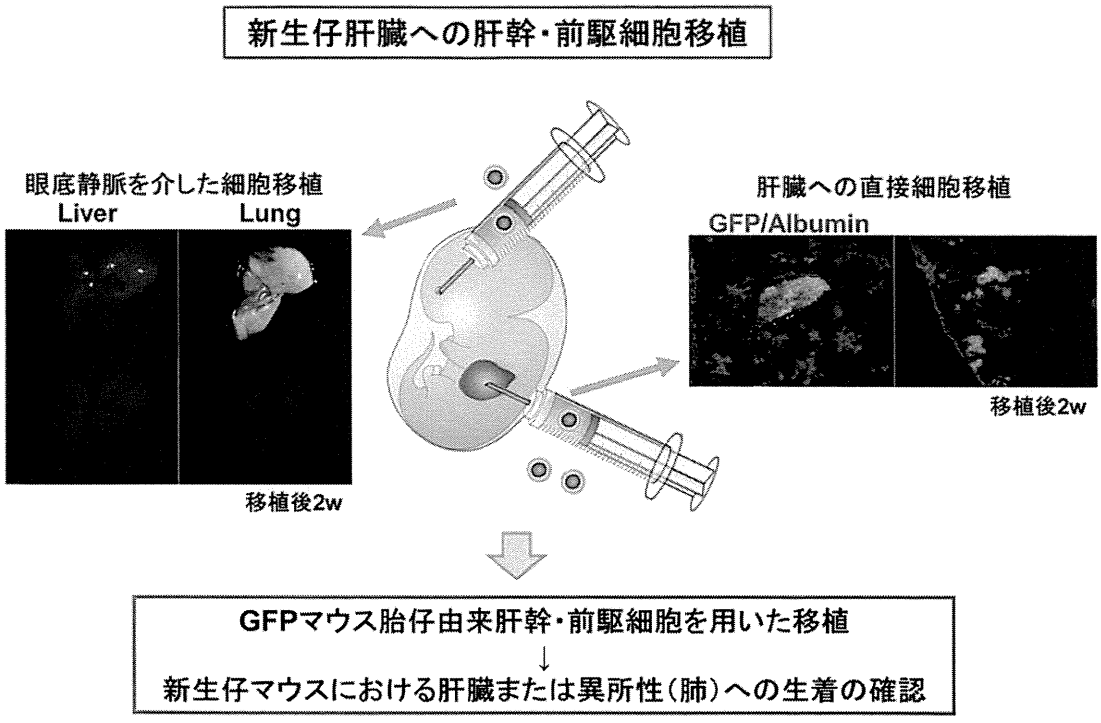


図9　肝幹・前駆細胞のマウス胎仔・新生仔への移植系

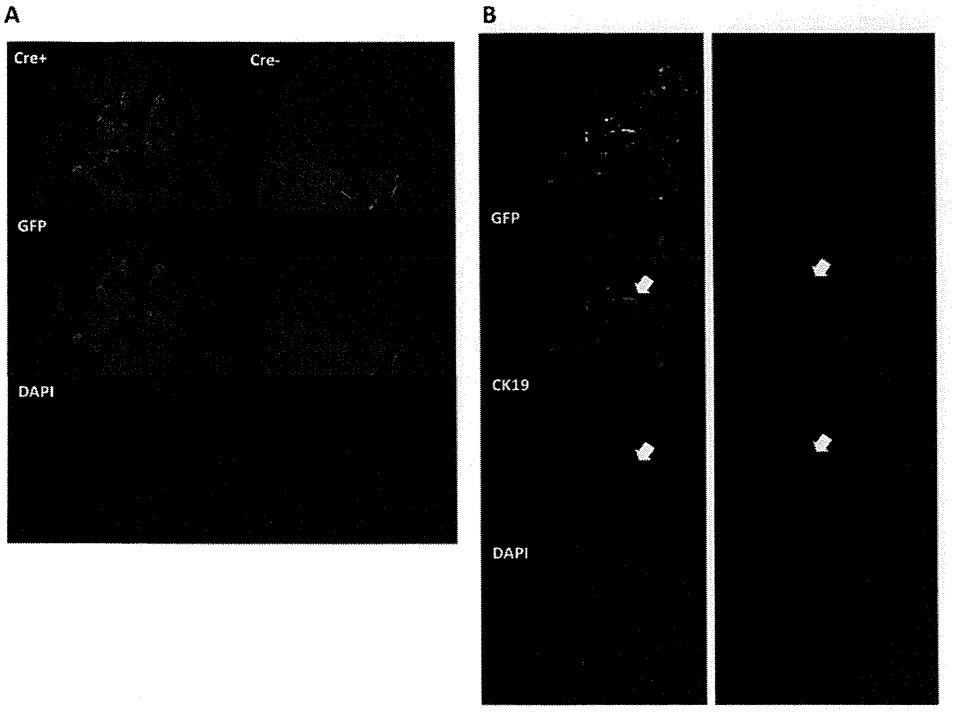


図10　肝幹・前駆細胞のマウス胎仔・新生仔への移植系

(A) Cre+/-マウスでの移植細胞の増殖　(B) 移植細胞での胆管マーカー（CK19）の発現

II. 研究に関する主な刊行物の一覧表

研究に関する主な刊行物の一覧

雑誌

原著論文

(1) Ito H, Kamiya A*, Ito K, Yanagida A, Okada K, Nakauchi H*. In vitro expansion and functional recovery of mature hepatocytes from mouse adult livers. Liver Int. 32(4):592-601. **2012** (*Corresponding Authors)

(2) Okada K, Kamiya A*, Ito K, Yanagida A, Ito H, Kondou H, Nishina H, Nakauchi H*. Prospective Isolation and Characterization of Bipotent Progenitor Cells in Early Mouse Liver Development. Stem Cells Dev. 21(7):1124-33. **2012** (*Corresponding Authors)

(3) Oikawa T, Kamiya A*, Zeniya M, Chikada H, Hyuck AD, Yamazaki Y, Wauthier E, Tajiri H, Miller LD, Wang XW, Reid LM*, Nakauchi H*. SALL4, a stem cell biomarker in liver cancers. Hepatology **57**, 1469-83, **2013**. (*Corresponding Authors)

(4) Yanagida A, Ito K, Chikada H, Nakauchi H, Kamiya A*. An in vitro expansion system for generation of human iPS cell-derived hepatic progenitor-like cells exhibiting a bipotent differentiation potential. PLoS One, 8, e67541, **2013** (*Corresponding Author)

総説

(1) 紙谷聡英「発生過程・生体における肝幹・前駆細胞の分化誘導の分子メカニズム」 肝胆膵, 2012, 65, 29-35

(2) 紙谷聡英「ヒト多能性幹細胞からの肝幹・前駆細胞分化誘導系の構築」 消化器内科, 2013, 56, 316-323

(3) 近田裕美、紙谷聡英「肝細胞移植を用いた肝疾患の治療」細胞 2013, 45, 314-317

(4) 及川恒一、紙谷聡英「幹細胞マーカーSALL4 の肝癌治療法における可能性」 肝胆膵, 2014, 68, 437-444

III. 研究に関する主な刊行物の別刷

Liver INTERNATIONAL
OFFICIAL JOURNAL OF THE INTERNATIONAL ASSOCIATION FOR THE STUDY OF THE LIVER

Liver International ISSN 1478-3223

BASIC STUDIES

In vitro expansion and functional recovery of mature hepatocytes from mouse adult liver

Hidenori Ito[1], Akihide Kamiya[1], Keiichi Ito[1], Ayaka Yanagida[1], Ken Okada[1] and Hiromitsu Nakauchi[1,2]

1 Division of Stem Cell Therapy, Center for Stem Cell Biology and Regenerative Medicine, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan
2 Exploratory Research for Advanced Technology, Japan Science and Technology Agency, Kawaguchi-shi, Saitama, Japan

Keywords
drug metabolism – *in vitro* expansion – mature hepatocyte

Abbreviations
2D, two-dimensional; 3D, three-dimensional; CK, cytokeratin; CPS, carbamoyl phosphate synthetase; CYP, cytochromes P450; DAPI, 4',6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFP, green fluorescent protein; GSK, glycogen synthase kinase; HGF, hepatocyte growth factor; HPRT, hypoxanthine phosphoribosyltransferase; MEF, mouse embryonic fibroblasts; MEK, MAPK/ERK kinase; PBS, phosphate-buffered saline; RT-PCR, reverse transcription-polymerase chain reaction; TAT, tyrosine aminotransferase; TGF, tumour growth factor.

Correspondence
Akihide Kamiya, PhD, and Hiromitsu Nakauchi, MD, PhD
Division of Stem Cell Therapy, Center for Stem Cell Biology and Regenerative Medicine, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokane-dai, Minato-ku, Tokyo 108-8639, Japan
Tel: +81 3 5449 5330
Fax: +81 3 5449 5451
e-mails: kamiyaa@ims.u-tokyo.ac.jp; nakauchi@ims.u-tokyo.ac.jp

Received 23 August 2011
Accepted 8 December 2011

DOI:10.1111/j.1478-3231.2011.02741.x

Abstract

Background: Mature hepatocytes retain the ability to regenerate the liver lobule fully *in vivo* following injury. Several cytokines and soluble factors (hepatocyte growth factors, epidermal growth factors, insulin and nicotinamide) are known to be important for proliferation of mature hepatocytes *in vitro*. However, hepatocytes monolayer-cultured on extracellular matrices have gradually lost their specific functions, particularly those in drug metabolism. *Aim:* We have explored and established a new culture system for expansion of functional hepatocytes. *Methods:* We evaluated two approaches for efficient expansion of mature hepatocytes: (i) Co-culture with mouse embryonic fibroblasts (MEF); (ii) Addition to culture of inhibitors of cell signals involved in liver regeneration. After expansion steps, 3-dimensional spheroid-forming culture was used to re-induce mature hepatocellular function. *Results:* The addition of inhibitors for tumour growth factor (TGF) β and glycogen synthase kinase (GSK) 3β efficiently induced *in vitro* expansion of mature hepatocytes. Although expression of hepatocellular functional genes decreased after expansion in monolayer culture, their expression and the activity of cytochrome P450 enzymes significantly increased with spheroid formation. Furthermore, when hepatocytes were co-cultured with MEF, addition of a MAPK/ERK kinase (MEK) inhibitor at the spheroid formation step enhanced drug-metabolism-related gene expression. *Conclusion:* Combination of the MEF co-culture system with the addition of inhibitors of TGFβ and GSK3β induced *in vitro* expansion of hepatocytes. Moreover, expression of mature hepatocellular genes and the activity of drug-metabolism enzymes in expanded hepatocytes were re-induced after spheroid culture.

The liver is an organ central to the maintenance of homeostasis *in vivo*. Hepatocytes, the main cellular component of the adult liver, express many enzymes and proteins required for mature liver functions, such as amino acid and lipid metabolism, gluconeogenesis, synthesis of serum proteins and xenobiotic detoxification. Cytochromes P450 (CYP, a superfamily of hemoproteins), catalyses the conversion of hydrophobic chemicals to more polar derivatives and is highly expressed in mature hepatocytes (1). The substrates of CYP include a wide variety of exogenous carcinogens and drugs as well as endogenous substrates, such as

steroid hormones or fatty acids. Primary hepatocytes and microsomes derived from hepatocytes are frequently used for the analysis of drug metabolism and hepatotoxicity. During liver regeneration *in vivo*, mature hepatocytes are temporally induced to divide with recovery of liver functions (2). Mature hepatocytes can also proliferate for a long time *in vivo*, as shown by serial transplantation of hepatocytes into mice with liver injury (3). Despite their high proliferation capacity *in vivo*, isolated hepatocytes lose viability and markers of hepatocellular differentiation within 3–4 days under normal culture condition (4, 5). Therefore, effective culture systems that maintain both proliferative activity and mature functions must be developed to permit expanded use of hepatocytes in pharmacologic analysis.

Several groups have studied culture systems that induce constitutive expansion of mature hepatocytes. Hepatocyte growth factor (HGF), epidermal growth factor (EGF) and tumor growth factor (TGF) α, which are important factors for *in vivo* liver regeneration, stimulate DNA synthesis in mature hepatocytes *in vitro* (6, 7). Addition of nicotinamide prolongs proliferation of mature hepatocytes (8, 9). In addition, a specific fraction of adult hepatocytes, known as small hepatocytes, were found to exhibit high proliferative activity and bi-potency for differentiation into hepatocytic and cholangiocytic cells (10, 11). However, after two-dimensional (2D) monolayer culture on several extracellular matrices, the functional characteristics of adult hepatocytes were down-regulated. When freshly isolated from livers, adult hepatocytes strongly expressed liver-enriched transcription factors (HNF1α, 1β, HNF3α, β, γ and HNF4α) for hepatocellular functional genes. Nevertheless, when these cells were cultured on collagen-coated dishes, expression of hepatocellular functional genes was significantly down-regulated because expression of these transcription factors fell during *in vitro* culture. Of interest is that addition of nicotinamide and matrigel to the culture partially induced expression of these genes (5, 7). In addition, primary hepatocytes cultured on moderately adhesive surfaces or cultured using hanging-drop technique spontaneously self-assemble into spherical aggregates ('spheroid formation'). Hepatocytes in the spheroids maintain expression of liver-enriched transcription factors and mature liver functions (12–14). In addition, several growth factors and dexamethasone induce expression of mature functional genes in 3D hepatic organoid culture (15). These results suggest that hepatocellular tertiary structure is important in maintenance of mature liver functions. However, we know of no study of de-differentiated hepatocytes that have recovered mature hepatocellular functions (i.e. CYP activity) on expansion using several growth factors.

In this study, we found that co-culture with mouse embryonic fibroblasts (MEF) or the addition to culture of inhibitors of cell signals modestly induced proliferation of mature hepatocytes from adult mouse livers. Activation of β-catenin signalling using inhibitor for glycogen synthase kinase (GSK) 3β and inhibition of TGFβ signalling significantly induced expansion of mature hepatocytes. Although these hepatocytes lost their mature hepatocellular functions during *in vitro* expansion, hepatocellular aggregation induced by hanging-drop culture permitted recovery of expression of several mature enzymes. In particular, spheroid formation in the presence of inhibitor of MAPK/ERK kinase (MEK) efficiently induced drug metabolic activity *in vitro*.

Materials and methods

Materials

C57BL/6NCrSlc mice and green fluorescent protein (GFP) transgenic mice (Nihon SLC, Shizuoka, Japan) were used in this study. All animals were treated under guidelines of the Institute of Medical Science, The University of Tokyo. Purchased reagents included Dulbecco's modified Eagle's medium (DMEM), DMEM/Ham's F12 half medium, penicillin/streptomycin/L-glutamine (100×), dexamethasone, nicotinamide, trypsin/EDTA, donkey serum and gelatin from porcine skin (Sigma, St Lois, MO, USA); insulin-transferrin-selenium X, non-essential amino acid solution, Hepes buffer solution and TRIzol (Invitrogen, Carlsbad, CA, USA); fetal bovine serum (FBS: Nichirei Biosciences, Tokyo, Japan); HGF and EGF (PeproTech, Rocky Hill, NJ, USA); collagen type I (Nitta Gelatin, Osaka, Japan); the selective MEK1/2 inhibitor N-[(2R)-2,3-dihydroxypropoxy]-3,4-difluoro-2-[(2-fluoro-4-iodophenyl)amino]-benzamide (PD0325901) and the selective GSK3β inhibitor 6-[[2-[[4-(2,4-dichlorophenyl)-5-(5-methyl-1H-imidazol-2-yl)-2-pyrimidinyl]amino]ethyl]amino]-3-pyridinecarbonitrile (CHIR99021: both Axon Biochemicals, Groningen, The Netherlands); the Rho-associated protein kinase inhibitor 4-[(1R)-1-aminoethyl]-N-4-pyridinyl-trans-cyclohexanecarboxamide, dihydrochloride (Y-27632; Wako Pure Chemicals, Osaka, Japan); the activin receptor like kinase (activated by TGFβ family) inhibitor A-83-01 (Tocris Bioscience, Bristol, UK) (16); Percoll solution (GE Healthcare UK, Amersham, UK).

Conditioned medium for *in vitro* expansion

To collect conditioned medium derived from mid-fetal mouse livers, we cultured E14 fetal liver cells dissociated using collagenase (17). Both parenchymal and non-parenchymal cells from fetal livers were cultured on gelatin-coated dishes. After 1 day of culture, non-attached hematopoietic cells were washed out with phosphate-buffered saline (PBS). Attached cells were cultured in DMEM supplemented with 10% FBS, 1× penicillin/streptomycin/L-glutamine, 1× non-essential amino acid solution, and 10^{-7} M dexamethasone. Conditioned media (3–5 day culture; 5–7 day culture) were collected and passed through 0.45 μm sterile filters.

Preparation and *in vitro* culture of mature hepatocytes from mouse livers

Hepatocytes and non-parenchymal cells were isolated from 6- to 8-weeks mouse livers following a 2-step collagenase digestion (18). Perfused liver tissues were dissociated with incubation in 0.05% collagenase solution for 10 min at 37°C. Mature hepatocytes were separated from non-parenchymal cells by several episodes of low-speed centrifugation (50× g, 1 min). Dead-cell debris was removed by centrifugation in 50% Percoll solution. Purified hepatocytes were counted using a hemocytometer. Cells were inoculated at a low density (3000 cells per one well in12-well culture plates) into dishes coated with type I collagen. For expansion assays with feeder cells, mitomycin-C treated-MEF were plated onto 0.1% gelatin-coated 12-well plates (2×10^5 cells per one well). After 24 h of culture, mature hepatocytes were inoculated onto feeder cells at a low density (3000 cells per one well in 12-well culture plates).

Our standard culture medium was a 1:1 mixture of H-CFU-C medium (DMEM/Ham's F12 half medium with 10% FBS, 1× insulin-transferrin-selenium X, 10 mM nicotinamide, 10^{-7} M dexamethasone, 2.5 mM HEPES, 1× penicillin/streptomycin/L-glutamine and 1× non-essential amino acid solution) and conditioned medium derived from E14 fetal liver cells (19, 20). For negative control without fetal liver-conditioned medium, we used a 1:1 mixture of H-CFU-C medium and non-conditioned medium. Cells were cultured for 6 day in standard culture medium in the presence of 40 ng/ml HGF and 20 ng/ml EGF.

Cell count analyses, adult hepatocytes cultured *in vitro*

Adult hepatocytes from GFP-transgenic mice were cultured on collagen-coated dishes or co-cultured with MEF. After 6 days of culture, cells were dissociated with 0.05% trypsin/EDTA for 5 min at 37°C. GFP-positive cells were counted using hemocytometer and fluorescence microscopy.

In several experiments, after 1 day or 6 days of culture, cells were fixed with 4% paraformaldehyde and nuclei were stained with 2 μg/ml 4′,6-diamidino-2-phenylindole (DAPI)/PBS. Nuclei in GFP-expressing cells were automatically counted using an ArrayScan VTI HCS Reader (Thermo Scientific, Waltham, MA, USA). Growth ratios were calculated (number of nuclei, 6 day culture/1 day culture).

Spheroid formation by expanded hepatocytes

After expansion of mature hepatocytes (co-cultured with MEF in the presence or absence of GSK3β and TGFβ inhibitors), cells were trypsinized. Dissociated cells were washed in DMEM with 10% FBS and counted. We placed 40-μl drops of hepatocyte culture medium (DMEM with 10% FBS, 1× non-essential amino-acid solution, 1× penicillin/streptomycin/L-glutamine, and 10^{-7} M dexamethasone) containing 1×10^4 hepatocytes on the inner aspects of the lids of 100 mm dishes containing PBS (to avoid desiccation) and cultured the cells in these hanging drops. In several experiments, 1 μM PD0325901 was added to the drop-culture medium. After 3 days of culture, spheroids of culture cells were collected and analysed.

mRNA detection by reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from hepatocytes using TRIzol. First-strand cDNA synthesized using a High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA) was used as a template for PCR amplification. For semi-quantitative RT-PCR, cDNA samples were normalized by number of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) copies, using the TaqMan probe (Life Technologies). Universal Library (Roche Diagnosis, Basel, Switzerland) was used for quantitative RT-PCR assays of CYP3A11, CYP3A16, tyrosine aminotransferase (TAT), carbamoyl phosphate synthetase (CPS) and hypoxanthine phosphoribosyltransferase (HPRT) transcripts. Intron-spanning primer sequences and probe number for each gene are shown in Table 1.

Immunocytochemistry and immunohistochemistry

For immunohistological analyses, adult hepatocellular spheroids were fixed with 4% paraformaldehyde in PBS for 12 h. Fixed spheroids snap-frozen in Optimal-Cutting-Temperature compound (Sakura Finetechnical, Tokyo, Japan) were sectioned at 6 μm onto glass slides. For immunocytochemical analyses, cultured hepatocytes were fixed with 4% paraformaldehyde in PBS for 15 min. Thawed sections or fixed cells were washed three times with PBS, and were permeabilized using 0.5% Triton X-100/PBS. After blocking with 5% donkey serum/PBS, samples were incubated overnight at 4°C with diluted primary antibodies [rabbit anti-mouse cytokeratin (CK) 19 antibody] (Prof. A. Miyajima, University of Tokyo, Tokyo, Japan); goat anti-mouse albumin antibody (Bethyl, Montgomery, TX, USA). The sections or cells next were washed three times with PBS and then were incubated for 1 h at room temperature with an Alexa488-conjugated donkey anti-rabbit IgG antibody (Invitrogen) and an Alexa546-conjugated donkey anti-goat IgG antibody (Invitrogen). Addition of appropriate IgG antibodies provided a negative control.

Analyses of CYP3A activity

CYP3A activity of hepatocytes was analysed using a P450-Glo™-CYP3A [luciferin-6′ pentafluorobenzyl-ether (PFBE) and luciferin-IPA] assay (Promega, Madison, WI, USA) according to the manufacturer's protocol.

Table 1. Primer list

HPRT	Fw	TCCTCCTCAGACCGCTTTT
	Rv	CCTGGTTCATCATCGCTAATC
	Probe number	95
CYP3A11	Fw	GGGACTCGTAAACATGAACTTTTT
	Rv	CCATGTCGAATTTCCATAAACC
	Probe number	53
CYP3A16	Fw	CTTCACAAACCGGCAGGA
	Rv	TCTCTTCCATTCCTCATCTTTAGC
	Probe number	(Only semi-quantitative RT-PCR)
TAT	Fw	GGAGGAGGTCGCTTCCTATT
	Rv	GCCACTCGTCAGAATGACATC
	Probe number	82
CPS	Fw	GACACCACTGCCCGAGAC
	Rv	CAGCAGACCTGCCACCTT
	Probe number	95
CK19	Fw	TGACCTGGAGATGCAGATTG
	Rv	CCTCAGGGCAGTAATTTCCTC
	Probe number	17
TGFβ1	Fw	AGCTCCACAGAGAAGAACTG
	Rv	ACCCACGTAGTAGACGATG
	Probe number	(Only semi-quantitative RT-PCR)
TGFβ2	Fw	CTCGAGGCGAGATTTGCAG
	Rv	TGTACCCTTTGGGTTCATGG
	Probe number	(Only semi-quantitative RT-PCR)
TGFβ3	Fw	CAACCCACACCTGATCCTC
	Rv	CAGAAGTTGGCATAGTAACCCTT
	Probe number	(Only semi-quantitative RT-PCR)

CK19, Cytokeratin 19; CPS, Carbamoyl-phosphate synthetase; CYP, Cytochrome P450; HPRT: Hypoxanthine phosphoribosyltransferase; TAT, Tyrosine aminotransferase.

Cells were cultured in 12-well-plates (monolayer culture) or 96-well-plates (spheroid culture). After 24 h exposure to 2×10^{-4} M dexamethasone, an inducer of CYP3A, cells were washed with PBS and cultured for 4 h in hepatocyte culture media containing luciferin-PFBE or for 1 h in hepatocyte culture media containing luciferin-IPA. Culture medium was transferred from each well to new tubes and an equal volume of detection reagent was added. After 20 min of incubation, CYP3A activity was detected using a luminometer.

Analyses of urea synthesis

Urea synthesis was analysed using a Urea Nitrogen Direct Kit (Stanbio Laboratory, Boerne, TX, USA) according to the manufacturer's protocol. Cells were cultured in 96-well-plates (spheroid culture). After 2 days incubation, medium was transferred from each well to new tubes, then BUN colour and acid reagents in this kit were added. After incubation, a red-purple chromogen was measured spectrophotometrically at 520 nm.

Statistics

We used Microsoft Excel 2007 software to calculate SD and statistically significant differences between samples using Student's 2-tailed *t* test.

Results

Co-culture with MEF and a combination of cell signal inhibitors induced efficient expansion of mature hepatocytes from adult mouse livers

We have recently found that co-culture with MEF significantly induces proliferation of fetal hepatocytes from early- to mid-fetal livers (Okada *et al.*, in press). The interaction between hepatocytes and MEF was important for effective expansion of fetal hepatocytes. We therefore addressed whether co-culture with MEF can induce proliferation of mature hepatocytes *in vitro*. Hepatocytes from GFP-transgenic mice were purified using low-speed centrifugation and inoculated onto collagen-coated dishes or MEF at a low density (3000 cells per one well, 12-well-dishes) and hepatocytes were counted. After 6 days, only culture with MEF yielded good numbers of large colonies (Fig. 1A). Co-culture with MEF significantly induced proliferation of adult hepatocytes (Fig. 1A, right panel). Interestingly, most of the colonies derived from mature hepatocytes contained both albumin-positive and CK19-positive cells, suggesting that part of cultured hepatocytes transdifferentiated into CK19-positive cholangiocytes *in vitro* (21).

Proliferation of hepatocytes was regulated by several soluble factors (HGF, EGF, and other cytokines) (6). In addition, TGFβ and Wnt are important for regulation of hepatocellular proliferation during *in vivo* liver regeneration (22–24). We therefore used specific inhibitors, to analyse these signal pathways activated by soluble factors. Inhibitors in the following factor/inhibitor pairs (MEK/PD0325901; TGFβ/A-83-01; GSK3β/CHIR99021; PI3K/LY294002, and Rock/Y-27632) were added into adult hepatocyte culture and hepatocytes were counted after 6 days of culture. The combination of GSK3β inhibitor and TGFβ inhibitor significantly induced proliferation of albumin-positive hepatocytes *in vitro* (Fig. 1B). We next compared the proliferative activity induced by MEF co-culture with that induced by the addition of inhibitors (Fig. 2). More cells were present in culture after supplementation with inhibitors than that after co-culture with MEF. In addition, proliferative activity of hepatocytes induced by the addition of signal inhibitors was not changed, regardless of the presence of feeder cells. These results suggest that inhibition of TGFβ signalling and activation of β-catenin signalling are important for effective expansion of mature hepatocytes *in vitro*.

Fetal liver-derived factors regulate proliferation of adult hepatocytes *in vitro*

Our conventional colony-expanding system for fetal hepatoblasts requires conditioned medium derived from mid-fetal liver cells (20, 25). We analysed the effect of fetal-liver conditioned medium on mature hepatocytes (Fig. 3A). When hepatocytes were cultured without conditioned medium, expansion of hepatocytes was

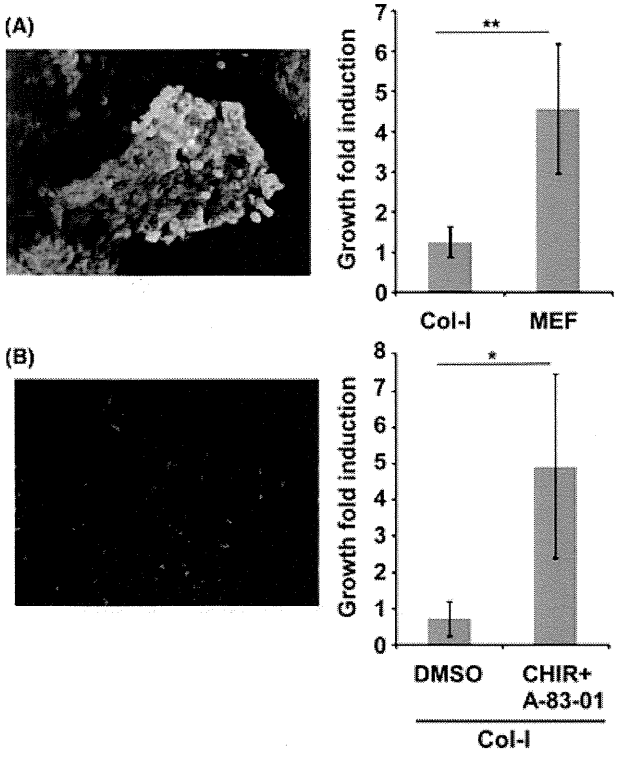


Fig. 1. Induction of proliferative activity in mature hepatocytes *in vitro*. (A) Hepatocytes from mouse adult livers were cultured on collagen-coated dishes (Col-I) or co-cultured with MEF (MEF) for 6 days. (left panel) Representative view of expanded hepatocytes co-cultured with MEF. (right panel) Relative proliferative activity of hepatocytes. The cell number after culture on collagen-coated dishes was defined as one. (B) Hepatocytes from mouse adult livers were cultured on collagen-coated dishes in the presence (CHIR+A-83-01) or absence (DMSO) of inhibitors for 6 days. (left panel) Representative view of expanded hepatocytes cultured with GSK3β and TGFβ inhibitors. (right panel) Relative proliferative activity of hepatocytes. The cell number after culture on collagen-coated dishes in the absence of inhibitors was defined as one. (A and B) Cells were stained with anti-albumin antibody (red) and anti-CK19 antibody (green). Results are represented as mean relative cell count ± SD (triplicate samples). $*P < 0.05$, $**P < 0.01$.

barely detected, suggesting that soluble factors derived from mid-fetal liver cells are important for proliferation of adult hepatocytes.

Fetal liver cells also expressed TGFβ1, TGFβ2 and TGFβ3 (Fig. 3B). TGFβ reportedly suppresses hepatocellular proliferation (26, 27). Fetal liver cells thus express both factors that induce and factors that inhibit proliferation of adult hepatocytes.

Expanded hepatocytes can re-express mature hepatocellular genes after spheroid formation

Mature hepatocytes have many metabolic functions and express enzymes subserving functions such as amino-acid synthesis and drug metabolism. TAT is important for amino-acid synthesis. CPS is a urea

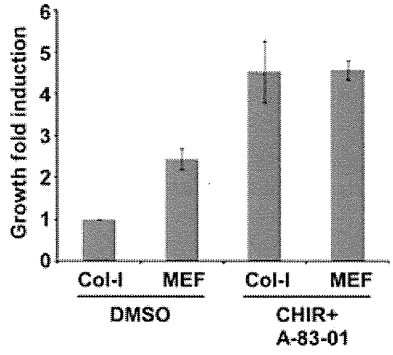


Fig. 2. Growth inductions in mature hepatocytes by co-culture with MEF and addition of GSK3β and TGFβ inhibitors. Hepatocytes from mouse GFP-transgenic mouse livers were cultured on collagen-coated dishes (Col-I) or co-cultured with MEF (MEF). GSK3β and TGFβ inhibitors were added into culture media (CHIR+A-83-01). DMSO: 0. 1% DMSO added (control for CHIR99021 and A-83-01). After 1 day and 6 days of culture, numbers of nuclei stained by DAPI in GFP-positive cells were counted automatically using ArrayScan. Growth ratio (number of nuclei, 6d:1d) on collagen-coated dishes in the absence of inhibitors was defined as one. Results are represented as mean growth ratio ± SD (duplicate samples).

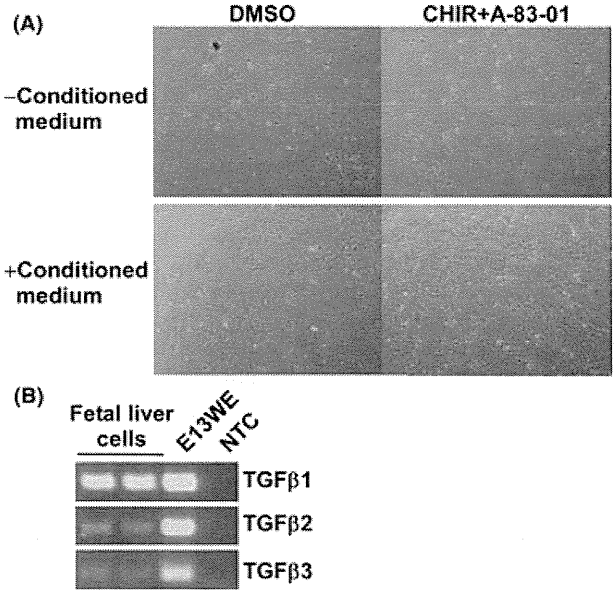


Fig. 3. Requirement of fetal liver-derived conditioned medium for expansion of mature hepatocytes *in vitro*. (A) Hepatocytes from adult livers were cultured with E14 fetal liver-derived conditioned medium (+Conditioned medium) or with non-conditioned medium (–Conditioned medium). Cells were cultured on collagen-coated dishes in the presence or absence of GSK3β and TGFβ inhibitors (CHIR+A-83-01) for 6 days. Under both conditions the addition of conditioned medium was strictly required for expansion of hepatocytes. DMSO: 0.1% DMSO added (control for CHIR99021 and A-83-01). (B) E14 fetal liver cells were cultured for 4 days and total RNA was purified. Expression of TGFβ family genes was detected using RT-PCR. E13WE, cDNA derived from E13 whole embryos. NTC, negative control without cDNA samples.

Liver International (2012)

cycle enzyme. CYP3A11 is the main enzyme for exogenous drug metabolism. Expression of these enzymes was significantly decreased after several days of 2D monolayer culture. As shown above, our culture system can induce proliferation of monolayer hepatocytes by co-culture with MEF or upon the addition of signalling inhibitors. After hepatocyte expansion, expression of mature hepatocellular genes (TAT, CPS and CYP3A11) also diminished in our culture system. To maintain functions of mature hepatocytes during long-term culture, three-dimensional (3D) biological structures are known to be important (12–14). Fresh suspended hepatocytes can self-assemble into 3D-compacted aggregates ('spheroids'). Hepatocytic spheroids exhibit mature liver functions; they also live longer than do cell maintained in monolayer culture. Therefore, we analysed whether expanded, de-differentiated hepatocytes can re-express particular functional enzymes after spheroid formation. Hepatocytes were cultured on collagen-coated dishes or on MEF in the presence or absence of GSK3β and TGFβ inhibitors. After 6 days of culture, these cells were dissociated and spheroid formation was induced using hanging-drop culture for 3 days (Fig. 4A). After 6 days of 2D-culture, expression of TAT, CPS and CYP3A11 was barely detected (Fig. 4B, 2D culture). For expansion of mature hepatocytes, we used three culture conditions (condition 1, addition of GSK3β and TGFβ inhibitors on collagen-coated dishes; 2, co-culture with MEF; 3, addition of GSK3β and TGFβ inhibitors and co-culture with MEF). Under all conditions, expanded hepatocytes re-expressed these enzymes after 3 days of spheroid formation culture (Fig. 4B, Spheroid). These results indicated that hepatocytes can recover their mature functions upon formation of 3D-structures after *in vitro* expansion and de-differentiation.

To improve culture condition for re-expression of mature hepatocellular genes, we analysed the effects of several inhibitors on the recovery step (spheroid formation culture) of hepatocellular function. The addition of the MEK inhibitor PD0325901 during spheroid formation significantly induced expression of CYP3A11 (Fig. 4B). Interestingly, the inducible effect of PD0325901 was detected only under culture with MEF (conditions 2 and 3), suggesting that interaction with MEF during monolayer culture is important for induction of CYP3A11 synthesis. Spheroids formed by expanded hepatocytes contained both albumin-positive hepatocytic cells and CK19-positive cholangiocytic cells (Fig. 5A). The addition of PD0325901 increased numbers of albumin-positive hepatocytic cells during spheroid formation. In contrast, expression of CK19 in spheroids was down-regulated by the addition of PD0325901 (Fig. 5B). Mature hepatocytes can trans-differentiate into CK19-positive cholangiocytic cells *in vitro* (21, 28). In our culture system, some expanded hepatocytes could differentiate into CK19-positive cells

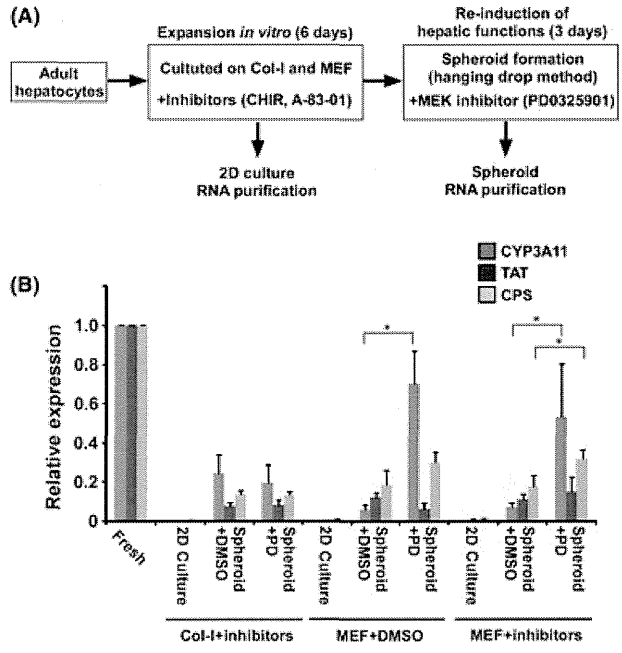


Fig. 4. Expression of hepatocellular functional genes was re-induced by the formation of spheroids. (A) Schema representing experimental method. Hepatocytes from adult mouse livers were cultured on collagen-coated dishes or co-cultured with MEF. In this expansion step (6 days of culture), GSK3β and TGFβ inhibitors were added in some experiments. After expansion, total RNA was purified from monolayer culture (2D culture samples). Expanded cells were trypsinized and spheroids were formed by hanging-drop culture. During this step, MEK inhibitor was added in some experiments. After 3 days of culture, total RNA was purified for RT-PCR analyses (Spheroid samples). (B) Expression of hepatocellular functional genes in 2D monolayer and 3D spheroid cultures of hepatocytes. Expansion of mature hepatocytes was induced by three conditions (condition 1, addition of GSK3β and TGFβ inhibitors on collagen-coated dishes, 2, co-culture with MEF, 3, addition of GSK3β and TGFβ inhibitors and co-culture with MEF). In several experiments, MEK inhibitor PD0325901 was added during spheroid formation. Expression of CYP3A11, CPS, and TAT was detected by quantitative RT-PCR. Expression of genes in hepatocytes freshly purified from adult mouse livers was set to 1.0. Results are represented as mean relative expression of genes ± SD (triplicate samples). $*P < 0.05$.

during monolayer culture (Fig. 1). The addition of PD0325901 thus may be capable of inducing re-differentiation of CK19-positive cells into functional hepatocytic cells during spheroid formation and may up-regulate expression of CYP3A11.

CYP3A activity is up-regulated by chemical inducers in re-differentiated hepatocytes

As shown above, *in vitro* expanded hepatocytes can re-differentiate and recover expression of the drug-metabolic enzyme, CYP3A11 during 3D culture. We analysed the activity of CYP3A in hepatocytic spheroids using luciferin-conjugated substrates (luciferin-PFBE).

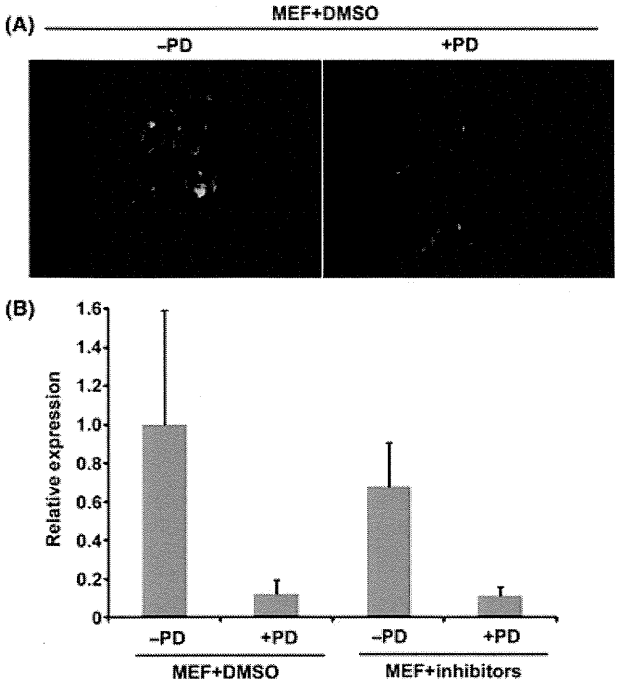


Fig. 5. MEK inhibitor induces hepatocytic differentiation of expanded hepatocytes during spheroid formation. Mature hepatocytes were co-cultured with MEF. GSK3β and TGFβ inhibitors or control DMSO was added. After 6 days of culture, cells were dissociated and spheroids were formed in the presence (+PD) or absence (−PD) of PD0325901 for 3 days. (A) Representative view of spheroid formed by expanded hepatocytes. Sections of spheroids were made and stained with anti-albumin (red) and anti-CK19 (green) antibodies. (B) Expression of CK19 in expanded hepatocyte-derived spheroids was analysed by quantitative RT-PCR. Results are represented as mean relative expression of CK19 ± SD (triplicate samples).

Mature hepatocytes were expanded under MEF co-culture for 6 days and hepatocellular enzymes were re-induced by spheroid formation. When freshly isolated hepatocytes were stimulated by high doses of dexamethasone, CYP3A activity was up-regulated. Similarly, spheroids formed from expanded hepatocytes responded to dexamethasone; the addition of dexamethasone induced CYP3A activity in spheroid culture (Fig. 6A). It has been reported that hepatocytes from neonatal and adult mice mainly express CYP3A16 and CYP3A11 respectively (29). We therefore analysed expression of both CYP3A family genes in our culture system and revealed that expanded hepatocytes in the spheroid culture mainly express CYP3A11 (Fig. 6B). In contrast, expression of CYP3A16 was only marginaly detected. Similar results were also obtained using more specific substrate for CYP3A, luciferin-IPA (Fig. 6C). These results clearly indicate that, under 3D culture conditions, expanded hepatocytes can recover not only expression of enzymes but also drug response activity.

Urea synthesis is partially induced in re-differentiated hepatocytes

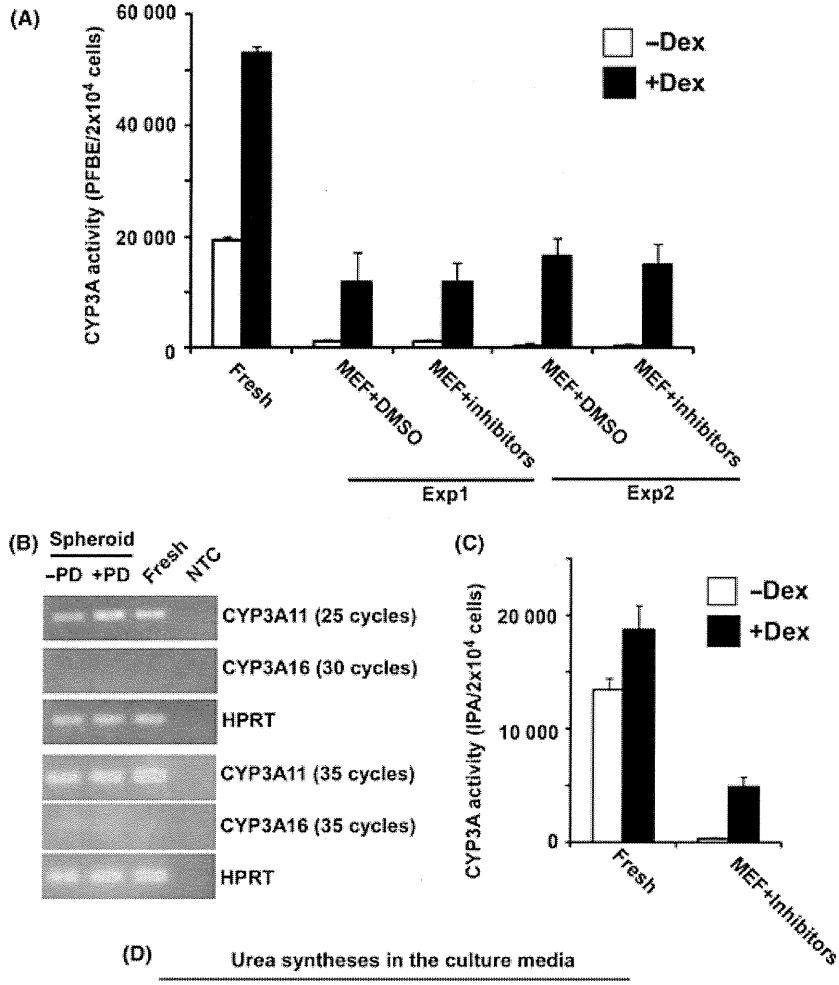
In addition to the drug-metabolic enzyme, *in vitro* expanded hepatocytes could recover expression of the urea cycle enzyme, CPS, during 3D culture. To further determine the activity of urea synthesis in our culture system, mature hepatocytes were expanded and hepatic differentiation was re-induced by spheroid formation. The results indicate that freshly isolated hepatocytes could produce huge amount of urea nitrogen in culture media after 2 days of culture. In comparison with fresh adult hepatocytes, however, production of urea in the spheroids from expanded hepatocytes was around 5% (Fig. 6D). Taken together, these results suggest that spheroid formation can partially re-induce the ability of urea synthesis in *in vitro* expanded hepatocytes.

Discussion

In this study, we found that both co-culture with MEF and the addition of inhibitors of GSK3β and TGFβ induced proliferation of adult hepatocytes *in vitro*. Although mature hepatocellular functions were decreased in these expanded hepatocytes, 3D spheroid formation in the presence of MEK inhibitors could re-induce expression of several mature hepatocellular genes and activation of CYP3A11. Interestingly, co-culture with MEF in expanding culture is required for induction of CYP3A11 synthesis by PD0325901 in our culture system (Fig. 4). The interaction between parenchymal cells and non-parenchymal cells is important for high-level functions of mature hepatocytes (30). It is possible that the interaction between expanded hepatocytes and MEF in spheroids induced the recovery of hepatocellular functions. During spheroid formation, cell-cell interaction between hepatocytes is mainly regulated by E-cadherin. In contrast, the molecular mechanism regulating the interactions between parenchymal hepatocytes and MEF remains unknown. In addition, to identify the mechanism by which inhibition of MEK leads to recovery of hepatocellular functions is an important issue. We found that the addition of MEK inhibitors decreased expression of CK19 in spheroids (Fig. 5). The effect MEK inhibitors on both expanded hepatocytes and MEF might regulate transdifferentiation of hepatocytes *in vitro*. Also possible is that MEK inhibitors directly induce expression of several hepatocellular genes, because expression of CYP2B in primary hepatocytes is induced by MEK inhibitors via the induction of nuclear translocation of constitutively active receptor (31). We speculate that several molecules downstream from MEK-MAPK in the MEK signalling pathway are involved in regulation of hepatocellular functions.

In other somatic tissues, stem and progenitor cells rather than terminally differentiated cells are the reservoir of proliferative activity and contribute to tissue regeneration. In contrast, progenitor-like proliferative

Liver International (2012)



(D) Urea syntheses in the culture media

Samples	Urea nitorgen (mg/dl)
MEF+2i (spheroid –PD)	0.227±0.051
MEF+2i (spheroid +PD)	0.254±0.085
Fresh hepatocytes	4.809±0.327

(1 x 10^4 cells / 2 days of culture)

Fig. 6. Activity of CYP3A and urea synthesis of hepatocytes cultured *in vitro*. Mature hepatocytes were co-cultured with MEF in the presence or absence of GSK3β and TGFβ inhibitors. After 6 days of expansion, cells were dissociated and spheroids were allowed to form in the 3 day hanging-drop culture. (A) CYP3A activities in expanded hepatocytes were detected using luciferin-PFBE. After hanging-drop culture in the presence of MEK inhibitor, spheroids were stimulated by the CYP3A inducer high-dose dexamethasone. Results are represented as mean CYP3A activity ± SD (n = 4). Results of two experiments are shown. (B) Expression of CYP3A11 and CYP3A16 in the spheroid culture derived from expanded hepatocytes. After hanging-drop culture in the presence (+PD/Spheroid) or absence (−PD/Spheroid) of MEK inhibitor, mRNA was purified and used for RT-PCR analyses. Fresh, freshly isolated hepatocytes; NTC, negative control without cDNA samples. (C) CYP3A activities in expanded hepatocytes were detected using luciferin-IPA. After hanging-drop culture in the presence of MEK inhibitor, spheroids were stimulated by the CYP3A inducer high-dose dexamethasone. Results are represented as mean CYP3A activity ± SD (n = 3). (D) Urea synthesis in the spheroid culture. After hanging-drop culture in the presence (+PD) or absence (−PD), spheroids were cultured in DMEM supplemented with 10% FBS, 1× penicillin/streptomycin/L-glutamine, 1× non-essential amino acid solution, and 10^{-7} M dexamethasone. After 2 days of culture, culture medium was analysed. Results are represented as mean urea nitrogen ± SD (n = 6).

activity is an important characteristic of adult hepatocytes, as shown *in vivo* (3). Proliferation of mature hepatocytes is mainly found during normal liver regeneration. However, several types of liver progenitor-like cells contribute to regeneration in severe liver injury. Adult livers contains two-types of liver progenitor-like

cells; $CD133^+$, $Ep\text{-}CAM^+$ hepatic progenitor cells and $CD44^+$ small hepatocytes (25, 32, 33). To exclude the possibility that these progenitors, as contaminants, proliferated in our culture studies, $CD133^+$ cells and $CD44^+$ cells were eliminated using magnetic beads. Most cells in the $CD133^-$ or $CD44^-$ fractions (the non-progenitor cells) could also proliferate in culture media with GSK3β and TGFβ inhibitors (data not shown). These results indicated that the proliferative activity of terminally differentiated hepatocytes was induced via the GSK3β/β-catenin and TGFβ signal pathways. Activation of β-catenin is important for the progression of liver regeneration (24). The analysis of molecular mechanism to induce hepatocellular proliferation by the signal pathways is important for establishment of systems effective in expanding adult hepatocytes *in vitro*.

We have shown that conditioned medium derived from E14 fetal liver cells is significantly effective for expansion of mature hepatocytes, indicating that some growth factors or cytokines other than HGF and EGF may be important for hepatocytic proliferation. Fetal livers contain both parenchymal hepatoblasts and several types of non-panrenchymal cells (*viz.*, mesothelial cells, fibroblasts and endothelial cells). Mesothelial cells and fibroblasts in fetal livers expressed midkine and pleiotrophin, which induce proliferation of fetal hepatocytes (34, 35). Using microarray expression analyses, we detected that Dlk^+CD133^+ fetal hepatoblasts also express several growth factors and cytokines inducing hepatocytic proliferation (A. Kamiya and H. Nakauchi, unpublished data). These results suggest that both parenchymal and non-parenchymal cells in fetal livers express soluble factors that are important for growth of hepatocytes. Identification of these soluble factors regulating hepatocellular proliferation and functionality will certainly facilitate induction of mature hepatocytes derived from pluripotent stem cells.

Acknowledgements

This study was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology in Japan and by an S-Innovation grant from Japan Science and Technology Agency.

Conflict of interest statement: There is no conflict of interest to disclose.

References

1. Cheung C, Gonzalez FJ. Humanized mouse lines and their application for prediction of human drug metabolism and toxicological risk assessment. *J Pharmacol Exp Ther* 2008; **327**: 288–99.
2. Fausto N, Campbell JS, Riehle KJ. Liver regeneration. *Hepatology* 2006; **43**(2 Suppl 1): S45–53.
3. Overturf K, Al-Dhalimy M, Ou CN, Finegold M, Grompe M. Serial transplantation reveals the stem-cell-like regenerative potential of adult mouse hepatocytes. *Am J Pathol* 1997; **151**: 1273–80.
4. Bissell DM, Guzelian PS. Phenotypic stability of adult rat hepatocytes in primary monolayer culture. *Ann N Y Acad Sci* 1980; **349**: 85–98.
5. Oda H, Nozawa K, Hitomi Y, Kakinuma A. Laminin-rich extracellular matrix maintains high level of hepatocyte nuclear factor 4 in rat hepatocyte culture. *Biochem Biophys Res Commun* 1995; **212**: 800–5.
6. Richman RA, Claus TH, Pilkis SJ, Friedman DL. Hormonal stimulation of DNA synthesis in primary cultures of adult rat hepatocytes. *Proc Natl Acad Sci U S A* 1976; **73**: 3589–93.
7. Block GD, Locker J, Bowen WC, *et al.* Population expansion, clonal growth, and specific differentiation patterns in primary cultures of hepatocytes induced by HGF/SF, EGF and TGF alpha in a chemically defined (HGM) medium. *J Cell Biol* 1996; **132**: 1133–49.
8. Inoue C, Yamamoto H, Nakamura T, Ichihara A, Okamoto H. Nicotinamide prolongs survival of primary cultured hepatocytes without involving loss of hepatocyte-specific functions. *J Biol Chem* 1989; **264**: 4747–50.
9. Sato F, Mitaka T, Mizuguchi T, Mochizuki Y, Hirata K. Effects of nicotinamide-related agents on the growth of primary rat hepatocytes and formation of small hepatocyte colonies. *Liver* 1999; **19**: 481–8.
10. Mitaka T. The current status of primary hepatocyte culture. *Int J Exp Pathol* 1998; **79**: 393–409.
11. Tateno C, Yoshizato K. Growth and differentiation in culture of clonogenic hepatocytes that express both phenotypes of hepatocytes and biliary epithelial cells. *Am J Pathol* 1996; **149**: 1593–605.
12. Koide N, Shinji T, Tanabe T, *et al.* Continued high albumin production by multicellular spheroids of adult rat hepatocytes formed in the presence of liver-derived proteoglycans. *Biochem Biophys Res Commun* 1989; **161**: 385–91.
13. Takezawa T, Yamazaki M, Mori Y, Yonaha T, Yoshizato K. Morphological and immuno-cytochemical characterization of a hetero-spheroid composed of fibroblasts and hepatocytes. *J Cell Sci* 1992; **101**(Pt 3): 495–501.
14. Lu HF, Chua KN, Zhang PC, *et al.* Three-dimensional coculture of rat hepatocyte spheroids and NIH/3T3 fibroblasts enhances hepatocyte functional maintenance. *Acta Biomater* 2005; **1**: 399–410.
15. Michalopoulos GK, Bowen WC, Mule K, Luo J. HGF-, EGF-, and dexamethasone-induced gene expression patterns during formation of tissue in hepatic organoid cultures. *Gene Expr* 2003; **11**: 55–75.
16. Tojo M, Hamashima Y, Hanyu A, *et al.* The ALK-5 inhibitor A-83-01 inhibits Smad signaling and epithelial-to-mesenchymal transition by transforming growth factor-beta. *Cancer Sci* 2005; **96**: 791–800.
17. Kamiya A, Kinoshita T, Ito Y, *et al.* Fetal liver development requires a paracrine action of oncostatin M through the gp130 signal transducer. *EMBO J* 1999; **18**: 2127–36.
18. Seglen PO. Hepatocyte suspensions and cultures as tools in experimental carcinogenesis. *J Toxicol Environ Health* 1979; **5**: 551–60.
19. Suzuki A, Zheng Y, Kondo R, *et al.* Flow-cytometric separation and enrichment of hepatic progenitor cells in the developing mouse liver. *Hepatology* 2000; **32**: 1230–9.

20. Kamiya A, Kakinuma S, Onodera M, Miyajima A, Nakauchi H. Prospero-related homeobox 1 and liver receptor homolog 1 coordinately regulate long-term proliferation of murine fetal hepatoblasts. *Hepatology* 2008; **48**: 252–64.
21. Nishikawa Y, Tokusashi Y, Kadohama T, Nishimori H, Ogawa K. Hepatocytic cells form bile duct-like structures within a three-dimensional collagen gel matrix. *Exp Cell Res* 1996; **223**: 357–71.
22. Oe S, Lemmer ER, Conner EA, *et al.* Intact signaling by transforming growth factor beta is not required for termination of liver regeneration in mice. *Hepatology* 2004; **40**: 1098–105.
23. Takiya S, Tagaya T, Takahashi K, *et al.* Role of transforming growth factor beta 1 on hepatic regeneration and apoptosis in liver diseases. *J Clin Pathol* 1995; **48**: 1093–7.
24. Tan X, Behari J, Cieply B, Michalopoulos GK, Monga SP. Conditional deletion of beta-catenin reveals its role in liver growth and regeneration. *Gastroenterology* 2006; **131**: 1561–72.
25. Kamiya A, Kakinuma S, Yamazaki Y, Nakauchi H. Enrichment and clonal culture of progenitor cells during mouse postnatal liver development in mice. *Gastroenterology* 2009; **137**: 1114–26, 26 e1-14.
26. Oberhammer FA, Pavelka M, Sharma S, *et al.* Induction of apoptosis in cultured hepatocytes and in regressing liver by transforming growth factor beta 1. *Proc Natl Acad Sci U S A* 1992; **89**: 5408–12.
27. Nakamura T, Tomita Y, Hirai R, *et al.* Inhibitory effect of transforming growth factor-beta on DNA synthesis of adult rat hepatocytes in primary culture. *Biochem Biophys Res Commun* 1985; **133**: 1042–50.
28. Nishikawa Y, Doi Y, Watanabe H, *et al.* Transdifferentiation of mature rat hepatocytes into bile duct-like cells *in vitro*. *Am J Pathol* 2005; **166**: 1077–88.
29. Li Y, Cui Y, Hart SN, Klaassen CD, Zhong XB. Dynamic patterns of histone methylation are associated with ontogenic expression of the Cyp3a genes during mouse liver maturation. *Mol Pharmacol* 2009; **75**: 1171–9.
30. Michalopoulos GK, Bowen WC, Zajac VF, *et al.* Morphogenetic events in mixed cultures of rat hepatocytes and nonparenchymal cells maintained in biological matrices in the presence of hepatocyte growth factor and epidermal growth factor. *Hepatology* 1999; **29**: 90–100.
31. Koike C, Moore R, Negishi M. Extracellular signal-regulated kinase is an endogenous signal retaining the nuclear constitutive active/androstane receptor (CAR) in the cytoplasm of mouse primary hepatocytes. *Mol Pharmacol* 2007; **71**: 1217–21.
32. Kon J, Ooe H, Oshima H, Kikkawa Y, Mitaka T. Expression of CD44 in rat hepatic progenitor cells. *J Hepatol* 2006; **45**: 90–8.
33. Schmelzer E, Zhang L, Bruce A, *et al.* Human hepatic stem cells from fetal and postnatal donors. *J Exp Med* 2007; **204**: 1973–87.
34. Asahina K, Tsai SY, Li P, *et al.* Mesenchymal origin of hepatic stellate cells, submesothelial cells, and perivascular mesenchymal cells during mouse liver development. *Hepatology* 2009; **49**: 998–1011.
35. Onitsuka I, Tanaka M, Miyajima A. Characterization and functional analyses of hepatic mesothelial cells in mouse liver development. *Gastroenterology* 2010; **138**: 1525–35.

STEM CELLS AND DEVELOPMENT
Volume 21, Number 7, 2012

DOI: 10.1089/scd.2011.0229

Prospective Isolation and Characterization of Bipotent Progenitor Cells in Early Mouse Liver Development

Ken Okada,[1,2] Akihide Kamiya,[1] Keiichi Ito,[1] Ayaka Yanagida,[1] Hidenori Ito,[1] Hiroki Kondou,[3] Hiroshi Nishina,[4] and Hiromitsu Nakauchi[1,5]

Outgrowth of the foregut endoderm to form the liver bud is considered the initial event of liver development. Hepatic stem/progenitor cells (HSPCs) in the liver bud are postulated to migrate into septum transversum mesenchyme at around embryonic day (E) 9 in mice. The studies of liver development focused on the mid-fetal stage (E11.5–14.5) have identified HSPCs at this stage. However, the in vitro characteristics of HSPCs before E11.5 have not been elucidated. This is probably partly because purification and characterization of HSPCs in early fetal livers have not been fully established. To permit detailed phenotypic analyses of early fetal HSPC candidates, we developed a new coculture system, using mouse embryonic fibroblast cells. In this coculture system, $CD13^+Dlk^+$ cells purified from mouse early fetal livers (E9.5 and E10.5) formed colonies composed of both albumin-positive hepatocytic cells and cytokeratin (CK) 19-positive cholangiocytic cells, indicating that early fetal $CD13^+Dlk^+$ cells have properties of bipotent progenitor cells. Inhibition of signaling by Rho-associated coiled-coil containing protein kinase (Rock) or by nonmuscle myosin II (downstream from Rock) was necessary for effective expansion of early fetal $CD13^+Dlk^+$ cells in vitro. In sorted $CD13^+Dlk^+$ cells, expression of the hepatocyte marker genes albumin and α-fetoprotein increased with fetal liver age, whereas expression of CK19 and Sox17, endodermal progenitor cell markers, was highest at E9.5 but decreased dramatically thereafter. These first prospective studies of early fetal HSPC candidates demonstrate that bipotent stem/progenitor cells exist before E11.5 and implicate Rock-myosin II signaling in their development.

Introduction

Liver development is regulated by hormonal factors as well as by cell–cell interaction. In the beginning of liver development, around embryonic day (E) 9 in mice, hepatic stem/progenitor cells (HSPCs) are believed to differentiate from foregut endoderm and to expand in the early fetal liver bud. Fibroblast growth factor derived from the cardiac mesoderm and bone-morphogenetic proteins from septum transversum mesenchyme are important for differentiation of foregut endodermal cells into hepatic-lineage cells [1,2]. During mid- to late-fetal development (around E11.5 to E16.5), hematopoietic stem cells originating from the aorta-gonad-mesonephros region migrate into the fetal liver. This implies significant change in the microenvironment of the liver from early- to late-fetal liver development. Oncostatin M secreted from hematopoietic cells induces hepatoblasts, hepatic progenitor cells in mid-fetal livers, to differentiate into mature hepatocytes [3,4]. Hepatoblasts can proliferate at high rate and possess bipotency, the ability to differentiate into both hepatocytes (hepatic parenchymal cells) and cholangiocytes [5]. Several groups have purified hepatoblasts derived from E11.5 to E14.5 livers. We have shown that a sorted individual cell derived from mid-fetal liver gives rise to a relatively large colony after 5 days of culture on extracellular matrix-coated dishes [6]. A member of this class of cells is designated as a "hepatic colony-forming unit in culture (H-CFU-C)." Utilizing H-CFU-C culture, delta-like 1 homolog (Dlk), Liv2, CD13, and CD133 were identified as cell surface markers for hepatoblasts [7–9]. H-CFU-C culture and reclone sorting have demonstrated that hepatoblasts in mid-fetal livers have self-renewal capacity and bipotency [10]. Several transcription factors, such as Prox1, Tbx3, and Sall4, regulate proliferation and differentiation of hepatoblasts in liver development [11–13].

[1]Division of Stem Cell Therapy, Center for Stem Cell Biology and Regenerative Medicine, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan.
[2]Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, Saitama, Japan.
[3]Department of Pediatrics, Graduate School of Medicine, Osaka University, Osaka, Japan.
[4]Department of Developmental and Regenerative Biology, Medical Research Institute, Tokyo Medical and Dental University, Tokyo, Japan.
[5]Exploratory Research for Advanced Technology, Japan Science and Technology Agency, Saitama, Japan.

In contrast to hepatoblasts in mid-fetal livers, few studies have been done with early fetal HSPCs in E9.5 and 10.5 liver buds (Fig. 1A), because no suitable culture system for these cells has been established. HSPCs in early fetal livers thus remain largely uncharacterized. Explant culture systems have been used to study early fetal liver cells, and the effects of fibroblast growth factor secreted from cardiac mesoderm on early fetal livers were found using explanted-liver organ culture [1,2]. However, as explanted early fetal livers do not consist solely of HSPCs, to establish a culture system for purified progenitor cells in such livers is crucial for analyses of the initial steps of liver development. In this study, we found that cells expressing CD13, Dlk, and Liv2 exist during early- to mid-fetal liver development. We established a new culture system for in vitro expansion of these cells, candidate HSPCs, at the single-cell level using mouse embryonic fibroblasts (MEFs) as feeder cells. $CD13^+Dlk^+$ cells derived from early fetal liver showed bipotency and could proliferate to form large colonies in this culture system. Inhibition of Rho-associated coiled-coil–containing protein kinase (Rock) or myosin II activity using, respectively, Y-27632 or blebbistatin significantly enhanced colony-forming activities of early fetal $CD13^+Dlk^+$ cells. This study, the first investigation of purified HSPCs derived from early fetal livers, demonstrates that these progenitor cells in early fetal livers have properties distinct from those in mid-fetal livers.

Materials and Methods

Materials

C57BL/6NCrSlc, C3H, and green fluorescent protein (GFP)-transgenic mice (Nihon SLC, Shizuoka, Japan) were used in this study. All animals were treated under guidelines of the Institute of Medical Science, The University of Tokyo. Reagents and commercial suppliers were Dulbecco's modified Eagle's medium (DMEM), DMEM/Ham's F12 half medium, penicillin/streptomycin/L-glutamine (100×), dexamethasone, dimethyl sulfoxide, nicotinamide, and gelatin from porcine skin (Sigma, St. Louis, MO); insulin–transferrin–selenium X, nonessential amino acid solution, KnockOut Serum Replacement (KSR), and HEPES buffer solution (Invitrogen, Carlsbad, CA); fetal bovine serum (FBS; Nichirei Biosciences, Tokyo, Japan); hepatocyte growth factor and epidermal growth factor (PeproTech, Rocky Hill, NJ); collagen type I (Nitta Gelatin, Osaka, Japan); PD0325901 and CHIR99021 (Axon Biochemicals, Groningen, The Netherlands); Y-27632 (Wako Pure Chemical Industries, Osaka, Japan); A-83-01 (Tocris Bioscience, Bristol, United Kingdom); and (S)-(−)-blebbistatin (Toronto Research Chemicals, Inc., Toronto, ON). Phycoerythrin (PE)-conjugated anti-CD13 (Pharmingen, San Jose, CA), fluorescein isothiocyanate (FITC)-conjugated anti-Dlk (Medical and Biological Laboratories, Nagoya, Japan), allophycocyanin (APC)-conjugated

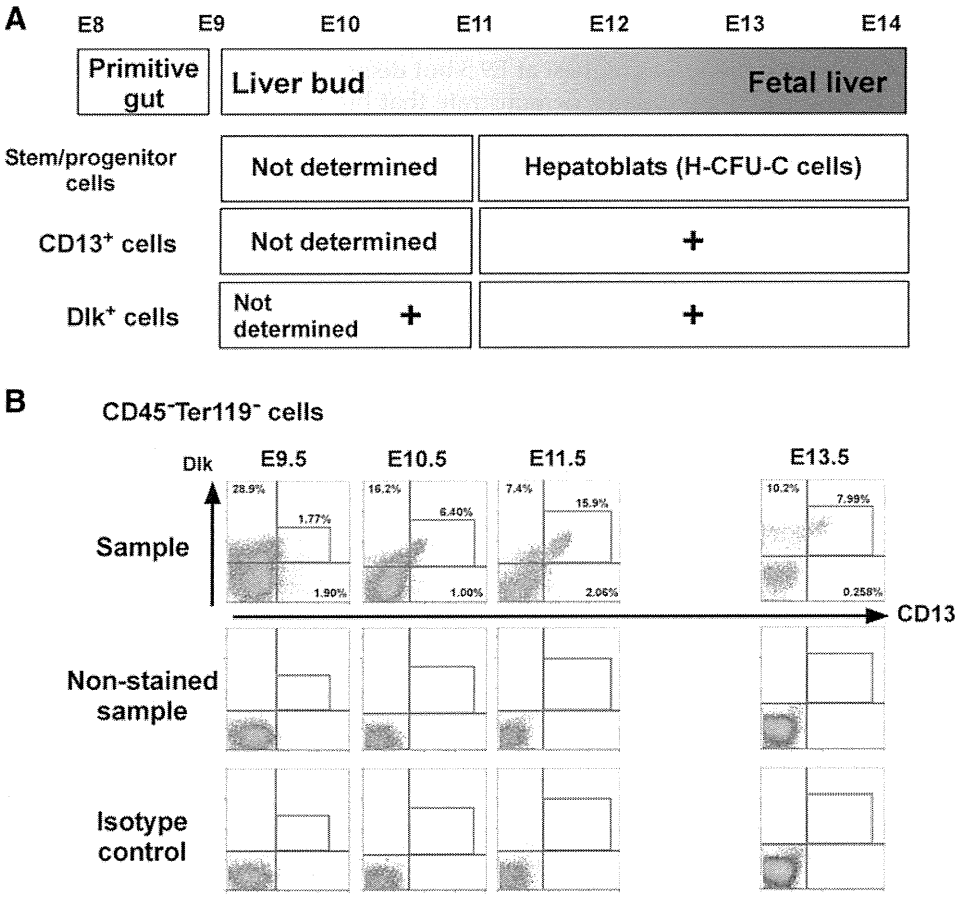


FIG. 1. Expression of cell surface markers in early- to mid-fetal liver cells. **(A)** Studies of hepatic stem/progenitor cells (HSPCs) during embryonic development. Bipotent progenitor cells in E11–14 mid-fetal livers (hepatoblasts) have been well characterized using H-CFU-C assays. In contrast, progenitor cells in early fetal livers (E9–10) have not been previously purified. **(B)** Cells from E9.5, E10.5, E11.5, and E13.5 fetal livers were stained with antibodies to various markers against mid-fetal hepatoblasts (CD13 and Dlk) and hematopoietic cells (CD45 and Ter119). Some populations of $CD45^-Ter119^-$ non-hematopoietic cells in early- and mid-fetal livers expressed both CD13 and Dlk. Nonstained cells (Nonstained sample), cells stained with isotype antibodies (Isotype control), or cells stained with anti-mouse CD13 and Dlk antibodies (Sample) were analyzed using flow cytometer. Ratios of CD13-single positive, Dlk-single positive, and $CD13^+Dlk^+$ cells in the $CD45^-Ter119^-$ fraction are shown. Numbers of $CD13^+Dlk^+$ cells in each of the $CD45^-Ter119^-$ fractions are as follows: 652 in 36,796 (E9.5), 1,630 in 25,481 (E10.5), 2,764 in 17,429 (E11.5), and 526 in 6,587 (E13.5). H-CFU-C, hepatic colony-forming unit in culture.

anti-Ter119, and APC-conjugated anti-CD45.2 (eBioscience, San Diego, CA) antibodies were used for cell staining.

Flow cytometric analysis

Minced liver tissues from E9.5 through E13.5 mice were dissociated with 0.05% collagenase solution. Dissociated cells were washed with phosphate-buffered saline (PBS) supplemented with 3% FBS and incubated with antibodies against cell surface markers for 60 min at 4°C. After washing with PBS supplemented with 3% FBS and staining of dead cells with propidium iodide, the cells were analyzed and sorted using a MoFlo™ fluorescence-activated cell sorter (DAKO, Glostrup, Denmark). Results with isotype control antibodies were shown as negative control.

Analysis of Liv2 expression using a fluorescence-activated flow cytometer

Dissociated cells were incubated with rat anti-Liv2 antibody [8] for 30 min on ice. After washing with PBS supplemented with 3% FBS, cells were stained with anti-rat immunoglobulin G (IgG)-Alexa647 (Invitrogen) for 30 min on ice and were washed with PBS supplemented with 3% FBS. Cells were further stained with FITC-conjugated anti-Dlk, PE-conjugated anti-CD13, PE-Cy7-conjugated anti-CD45, and PE-Cy7-conjugated anti-Ter119 (eBioscience) antibodies for 30 min on ice. After washing with PBS supplemented with 3% FBS and staining of dead cells with propidium iodide, the cells were analyzed and sorted using a MoFlo fluorescence-activated cell sorter.

Preparation of MEF

E13.5 ICR mouse embryos (Nihon SLC) were dissected and the head and internal organs were completely removed. The torso was minced and dissociated in 0.05% trypsin–EDTA (Sigma) for 30 min. After washing with PBS, cells were expanded in DMEM with 10% FBS. To halt cell proliferation, these MEFs were treated with mitomycin C (Wako Pure Chemical Industries) at 37°C for 2 h and used as feeder cells.

Colony formation assay

$CD13^+Dlk^+$ and other types of cells in the nonhematopoietic cell fraction were plated onto type I collagen or gelatin-coated 35-mm tissue culture dishes at a low density (25 cells/cm^2) or into type I collagen-coated 96-well plates at one cell per well. For colony formation assay with feeder cells, mitomycin C-treated feeder cells (MEF or other cell lines) were plated onto 0.1% gelatin-coated 12-well plates (2×10^5 cells per well). After 24 h of culture, cells in the nonhematopoietic cell fraction were sorted onto feeder cells at a low density (25 cells/cm^2).

Our standard culture medium is a 1:1 mixture of H-CFU-C medium (DMEM/Ham's F12 half medium with 10% FBS or 10% KSR, 1× Insulin–Transferrin–Selenium X, 10 mM nicotinamide, 10^{-7} M dexamethasone, 2.5 mM HEPES, 1× penicillin/streptomycin/L-glutamine, and 1× nonessential amino acid solution) and conditioned medium derived from E14.5 hepatic cells [13]. In several experiments, we used a 1:1 mixture of H-CFU-C medium and fresh DMEM with 10% KSR as a culture medium for coculture colony assays. Cells were cultured for 6 days, in the presence of 40 ng/mL hepatocyte growth factor and 20 ng/mL epidermal growth factor, in either standard culture medium or 1:1 mixture of H-CFU-C medium and fresh DMEM with 10% KSR. To count colonies derived from individual single cells, we used an ArrayScan VTI HCS Reader (Thermo Scientific, Waltham, MA) following immunostaining. Albumin-positive colonies were counted.

To analyze the effects of Rock-myosin II pathway using inhibitors, $CD45^-Ter119^-Dlk^+$ cells derived from GFP transgenic mouse embryos were cocultured with MEF in the presence of either Y-27632 or blebbistatin. GFP-positive colonies were counted. To analyze the effects of soluble factors derived from MEF, MEF-conditioned medium was harvested from 2-day confluent cultures of MEF cultured in a 1:1 mixture of H-CFU-C medium and fresh DMEM with 10% KSR. We cultured early fetal cells on collagen-coated dishes or on MEF feeder cells in MEF-conditioned medium.

Messenger RNA detection by reverse transcription–polymerase chain reaction

Total RNA was extracted from $CD13^+Dlk^+$ cells in the nonhematopoietic cell fraction using the RNeasy Micro Kit (Qiagen, Venlo, The Netherlands). First-strand cDNA synthesized using the Primescript first strand cDNA synthesis kit (Takara, Otsu, Japan) was used as a template for quantitative reverse transcription–polymerase chain reaction (RT-PCR) amplification. The cDNA samples were normalized by number of glyceraldehyde 3-phosphate dehydrogenase copies using quantitative RT-PCR with the TaqMan probe (Applied Biosystems, Foster City, CA). Universal Library (Roche Diagnosis, Basel, Switzerland) was used to quantify the copy numbers of albumin, α-fetoprotein (AFP), cytokeratin (CK) 19, c-Met, E-cadherin, and Sox17 transcripts. Intron-spanning primer sequences and probe number for each gene are shown in Supplementary Table S1 (Supplementary Data are available online at www.liebertonline.com/scd).

For hepatic gene expression analyses in HSPC colonies, E10.5 and E13.5 $CD13^+Dlk^+$ cells derived from GFP-transgenic mice were cultured with MEF for 3 days. Colonies were dissociated in 0.05% trypsin–EDTA and GFP^+ cells were purified using a MoFlo fluorescence-activated cell sorter. Total RNA was extracted and first-strand cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Expression of hepatic genes was detected using quantitative RT-PCR with the TaqMan probe: Cyp1A1, Mm00487218_m1; Cyp1A2, Mm00487224_m1; Cyp3A11, Mm00731567_m1; Cyp3A13, Mm00484110_m1; Cyp7a1, Mm00484152_m1; glucose-6-phosphatase, Mm00839363_m1; tyrosine aminotransferase, Mm01244282_m1; albumin, Mm00802090_m1; CK19, Mm00492980_m1.

Immunostaining

Cultured cells were washed with PBS and were fixed with 4% paraformaldehyde/PBS. After washing 3 times with PBS, cells were permeabilized with 0.5% Triton/PBS for 10 min, washed with PBS, and incubated with 5% donkey serum/PBS for 1 h at room temperature. They were incubated with diluted primary antibodies overnight at 4°C. Goat anti-albumin antibody (Bethyl, Montgomery, TX) and rabbit anti-CK19 antibody (A gift from Prof. A. Miyajima, University of

Tokyo, Tokyo, Japan) were used as primary antibodies [7]. The cells next were washed with PBS and were incubated for 1 h at room temperature with anti-rabbit IgG-Alexa488 and anti-goat IgG-Alexa546 antibodies (Invitrogen). The cells were washed with PBS and their nuclei were stained with 40,6-diamidine-20-phenylindole dihydrochloride (Sigma).

For the analyses of proliferation, colonies were stained with goat anti-albumin antibody and rabbit anti-Ki67 antibody (Abcam, Cambridge, United Kingdom). After washing with PBS, cells were stained with anti-rabbit IgG-Alexa555 and anti-goat IgG-Alexa488 antibodies (Invitrogen). Intensities of Ki67 in individual albumin-positive colonies were analyzed with an ArrayScan VTI HCS Reader.

In-droplet cell-staining methods

To quantify albumin and CK19 expression in individual cells, in-droplet staining methods were used [14]. $CD13^+$ Dlk^+ cells derived from fetal livers were sorted onto slide glasses. After fixation with 2% paraformaldehyde/PBS and permeabilization with 0.5% Triton/PBS, cells were incubated in 5% donkey serum/PBS for 30 min at room temperature. They then were incubated with goat anti-albumin and rabbit anti-CK19 antibodies overnight at 4°C, washed with PBS, and incubated with secondary antibodies (anti-rabbit IgG-Alexa488 and anti-goat IgG-Alexa546 antibodies) for 40 min at room temperature. The cells were washed with PBS and their nuclei were stained with 40,6-diamidine-20-phenylindole dihydrochloride. For each analysis, addition of an appropriate immune serum provided a negative control. Antibody fluorescence intensity was measured using the ArrayScan Reader.

Statistics

We used Microsoft Excel 2004 for Mac, Version 11.6.2 (Microsoft, Redmond, WA) to calculate standard deviations (SDs) and statistically significant differences between samples using Student's 2-tailed *t*-test.

Results

Early fetal liver contains cells expressing mid-fetal hepatoblast cell-surface markers

Several studies show that CD13 and Dlk are cell surface markers of hepatoblasts in mid-fetal livers (E11.5 to E14.5) and hepatoblasts exist in the $CD13^+Dlk^+$ fraction [7,15]. We assessed whether early fetal livers (E9.5 and E10.5) contain cells expressing these cell-surface markers (Fig. 1B). Livers derived from E9.5 to E13.5 mouse embryos were dissected and dissociated using collagenase. Cells were stained with antibodies against hematopoietic cell surface markers (CD45 and Ter119) as well as CD13 and Dlk. $CD13^+Dlk^+$ double-positive cells were found in the $CD45^-Ter119^-$ nonhematopoietic cell fraction derived from both early- and mid-fetal livers, although the expression level of CD13 and Dlk was low at E9.5 and increased during liver development. Liv2 is another cell surface molecule expressed on hepatic progenitor cells; numbers of Liv2-positive cells increase during E9.5 to E12.5 [8]. We found that $CD13^+Dlk^+$ cells in E9.5 fetal liver also expressed Liv2, indicating that cells expressing several hepatoblast cell-surface markers existed during early- to mid-fetal liver development (Supplementary Fig. S1). These results suggested that cell surface markers of hepatoblasts are commonly encountered during fetal liver development.

Phenotypic differences between early- and mid-fetal $CD13^+Dlk^+$ cells

To further characterize $CD13^+Dlk^+$ cells from each stage, expression of endodermal, hepatocyte, and cholangiocyte marker genes was analyzed using real-time RT-PCR. Strong expression of albumin and AFP was detected in $CD13^+Dlk^+$ cells derived from E13.5 mid-fetal liver (Fig. 2A). In contrast, these hepatocyte marker genes were detected at low levels in $CD13^+Dlk^+$ cells derived from E9.5 early fetal liver. Interestingly, E9.5 $CD13^+Dlk^+$ cells expressed CK19 and the early endodermal cell marker Sox17 at high levels (Fig. 2B). Although CK19 is known as a marker for cholangiocytes, it is also expressed in primitive gut endoderm at an earlier stage, including E9.0 [16]. Synthesis of albumin and CK19 was also analyzed using an in-droplet staining method (Fig. 2C). Although albumin levels were barely detectable in E9.5 $CD13^+Dlk^+$ cells, but they were accumulated during liver development. In contrast, CK19 expression levels in $CD13^+$ Dlk^+ cells derived from E9.5 and E10.5 livers were higher than those in $CD13^+Dlk^+$ cells derived from E11.5 and E13.5 livers. These results indicate that gene expression patterns in early fetal $CD13^+Dlk^+$ cells are distinct from those in mid-fetal $CD13^+Dlk^+$ cells.

Proliferative capacity of $CD13^+Dlk^+$ cells derived from early- and mid-fetal livers

To analyze whether early fetal $CD13^+Dlk^+$ cells contain phenotypes of hepatic progenitor cells (exhibiting high proliferative potential and bipotency), we analyzed these cells using single-cell colony assays. Sorted single cells were inoculated into individual wells of 96-well collagen type I-coated culture plates. Cells derived from mid-fetal livers (E11.5 and E13.5) formed several small colonies (50–100 cells) and large colonies (over 100 cells) after 6 days of culture [13]. In contrast, cultures derived from E9.5 and E10.5 livers yielded few colonies or none (Supplementary Fig. S2). The nonhematopoietic cell fraction of E9.5 fetal livers comprises almost 1% $CD13^+Dlk^+$ cells and 99% $CD13^-$ or Dlk^- cells (Supplementary Fig. S3A). To exclude the possibility that early fetal progenitor cells are in the Dlk^- or $CD13^-$ fraction, we sorted 10,000 E9.5 fetal liver cells (100 $CD13^+Dlk^+$ cells and 9,900 $CD13^-$ or Dlk^- cells). Cells from neither fraction could form colonies in H-CFU-C culture medium on collagen-coated dishes. In addition, no colonies were detected in cultures derived from 10,000 nonsorted E9.5 liver cells, indicating that low colony-forming activities of E9.5-derived cells were not due to damage sustained during flow cytometry (Supplementary Fig. 3B). Nonsorted cells derived from E10.5 livers could form only a few small colonies but not large colonies (Supplementary Fig. 3C). Few colony formation of early fetal livers was also detected on gelatin-coated dishes (data not shown). These results suggested that conventional H-CFU-C culture on collagen- and gelatin-coated dishes is not suitable for early fetal (E9.5–10.5) HSPCs.

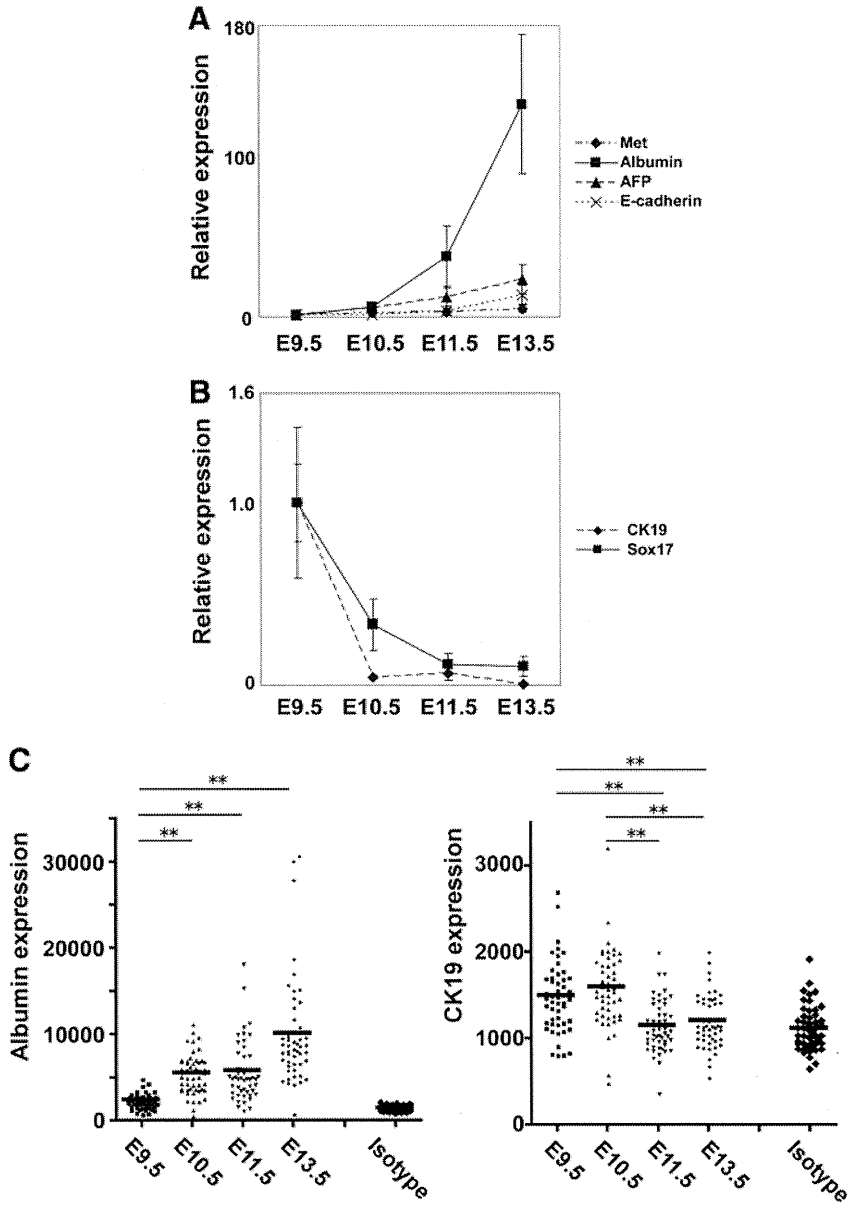


FIG. 2. Gene expression profiles of $CD45^-Ter119^-CD13^+Dlk^+$ cells during fetal liver development. **(A, B)** Expression of several hepatocyte (Met, albumin, AFP, and E-cadherin), primitive gut endoderm and cholangiocyte (CK19), and endodermal progenitor (Sox17) markers was analyzed using quantitative reverse transcription–polymerase chain reaction. $CD45^-Ter119^-CD13^+Dlk^+$ cells derived from E9.5, E10.5, E11.5, and E13.5 fetal livers were purified using flow cytometry. Expression level of marker genes in E9.5 cells was set to 1.0. Results are represented as relative expression ± SD (triplicate samples). **(C)** Protein expression (albumin and CK19) in $CD45^-Ter119^-CD13^+Dlk^+$ cells derived from E9.5, 10.5, 11.5, and E13.5 fetal livers. $CD45^-Ter119^-CD13^+Dlk^+$ cells were directly sorted onto slide glasses and were stained with primary antibodies (goat anti-albumin and rabbit anti-CK19 antibodies) and secondary antibodies (anti-rabbit IgG-Alexa488 and anti-goat IgG-Alexa546 antibodies). Results are represented as intensities of fluorescence of randomly picked-up cells ($n = 50$, $^{**}P < 0.01$). CK, cytokeratin; IgG, immunoglobulin G; AFP, α-fetoprotein.

Early fetal liver $CD13^+Dlk^+$ cells require both feeder cell interaction and the addition of ROCK inhibitor for their optimal expansion

Several studies have suggested that cell–cell interactions are important for proliferation and differentiation of somatic stem cells and progenitor cells. In early fetal liver development, the interaction between endodermal and mesenchymal populations is important for proper liver bud growth. Transcription factor Hlx, expressed in the septum transversum mesenchyme, is essential for proliferation of early fetal hepatic cells [17]. Therefore, we inferred that E9.5 $CD13^+Dlk^+$ cells need to interact with other cell populations to propagate both in vivo and in vitro. To mimic the interaction of hepatic and mesenchymal cells, $CD13^+Dlk^+$ cells were cocultured with MEF as mesenchymal feeder cells. We sorted either $CD13^+Dlk^+$ cells or other cells (Dlk^- or $CD13^-$ cells) in the non-hematopoietic cell fraction of E9.5 and E13.5 fetal livers. After 6 days of coculture with MEF, a few large colonies (containing both $albumin^+$ hepatocytic cells and $CK19^+$ cholangiocytic cells) were detected in culture of E9.5 $CD13^+Dlk^+$ cells (Fig. 3A). Some early fetal $CD13^+Dlk^+$ cells possess phenotypes of hepatic progenitor cells, *viz.*, high proliferative activity and bipotency. In contrast to mid-fetal hepatoblasts, early fetal HSPCs required interaction with mesenchymal fibroblasts for in vitro expansion. After 3 days of colony formation culture, early fetal $CD13^+Dlk^+$ cells expressed several hepatic genes and their expression levels were lower than those in mid-fetal $CD13^+Dlk^+$ cells, suggesting that early fetal cells are more immature types of progenitors (Supplementary Table S2). KSR is routinely employed in serum-free embryonic stem-cell culture protocols. We found that numbers of large and small colonies derived from E9.5 $CD13^+Dlk^+$ cells detected in KSR supplemented culture increased compared with those detected in FBS-supplemented culture (Fig. 3B). To explore signaling pathways regulating proliferation of early fetal liver