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An Optimal Medium Supplementation Regimen for Initiation of Hepatocyte Differentiation in Human Induced Pluripotent Stem Cells

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ABSTRACT

Human induced pluripotent stem (hiPS) cells are an ideal source for hepatocytes. Glucose and arginine are necessary for cells to survive. Hepatocytes have galactokinase (GALK), which metabolizes galactose for gluconeogenesis, and ornithine transcarbamylase (OTC), which converts ornithine to arginine in the urea cycle. Hepatocyte selection medium (HSM) lacks both glucose and arginine, but contains galactose and ornithine. Although human primary hepatocytes survive in HSM, all the hiPS cells die in 3 days. The aim of this study was to modify HSM so as to initiate hepatocyte differentiation in hiPS cells within 2 days. Hepatocyte differentiation initiating medium (HDI) was prepared by adding oncostatin M (10 ng/ml), hepatocyte functional proliferation inducer (10 nM), 2,2′-methylenebis (1,3-cyclohexanedione) (M50054) (100 μg/ml), 1× non-essential amino acid, 1× sodium pyruvate, nicotinamide (1.2 mg/ml), L-proline (30 ng/ml), and L-glutamine (0.3 mg/ml) to HSM. HiPS cells (201B7 cells) were cultured in HDI for 2 days. RNA was isolated, used as template for cDNA, and subjected to real-time quantitative polymerase chain reaction. Alpha-fetoprotein, γ-glutamyl transpeptidase, and delta-like 1 were upregulated. Expression of albumin was not observed. Expression of transcription factors specific to hepatocytes was upregulated. The expression of GALK2, OTC, and CYP3A4 were increased. In conclusion, differentiation of 201B7 cells to hepatoblast-like cells was initiated in HDI. Limitations were small number of cells were obtained, and the cells with HDI were not mature hepatocytes. J. Cell. Biochem. 116: 1479–1489, 2015.

KEY WORDS: APOPTOSIS INHIBITOR; ONCOSTATIN M; REAL-TIME QUANTITATIVE PCR

Acute liver failure, in most cases, is fatal and requires liver transplantation for the patients to survive [Sugawara et al., 2012]. Hepatocyte transplantation can serve as an alternative when there is a shortage of organ donors. Primary hepatocytes can be transplanted to patients with liver failure [Strom et al., 1999]. However, primary hepatocyte culture remains technically difficult. Hepatocytes are isolated from a fragment of resected donor liver with a 2-step collagenase perfusion [Strom et al., 1999]. Isolated hepatocytes are prone to apoptosis and damage [Fisher et al., 2004] and have difficulty in proliferation once cultured [Mitaka et al., 1991]. Primary

Conflict of interest: The authors have nothing to declare.

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TABLE I. Primers of Real-Time Quantitative Polymerase Chain Reaction

	Sequence	Description	Product size	GenBank
OMC311	CCGTTTTTGGCTCTGTTTTG	qPCR, hNanog, forward	187 bp	NM_024865
OMC312	TCATCGAAACACTCGGTGAA	qPCR, hNanog, reverse		
OMC317	ACACAAAAAGCCCACTCCAG	qPCR, hAFP, forward	147 bp	NM_001134
OMC318	GGTGCATACAGGAAGGGATG	qPCR, hAFP, reverse		
OMC321	CGAATGCCAGAGAAGGTCAC	qPCR, hRPL19, forward	157 bp	BC000530
OMC322	CCATGAGAATCCGCTTGTTT	qPCR, hRPL19, reverse		
OMC329	GCTCGTGAAACACAAGCCCAAG	qPCR, hAlbumin, forward	114 bp	NM_000477
OMC330	GCAAAGCAGGTCTCCTTATCGTC	qPCR, hAlbumin, reverse		
OMC347	GCCCAGTGAACAGAATAAAGGTGC	qPCR, hHEX, forward	167 bp	NM_002729
OMC348	CCAATGCCAGTGGTCATCATCC	qPCR, hHEX, reverse		
OMC349	TCTCAGTCAGTGCGATGTCTGG	qPCR, hGATA4, forward	197 bp	NM_002052
OMC350	AGGAGGGAAGAGGGAAGATTACG	qPCR, hGATA4, reverse		
OMC351	CGGACTTGGTGCGTCTAAGATG	qPCR, hCEBPA, forward	148 bp	U34070
OMC352	GCATTGGAGCGGTGAGTTTG	qPCR, hCEBPA, reverse		
OMC367	GGATGAGTGCGTCATAGCAA	qPCR, hDlk-1, forward	121 bp	NM_005618
OMC368	CCTCCTCTTCAGCAGCATTC	qPCR, hDlk-1, reverse		
OMC385	CCTCATCCTCAACATCCTCAAAGG	qPCR, hG-GTP, forward	163 bp	J04131
OMC386	CACCTCAGTCACATCCACAAACTTG	qPCR, hG-GTP, reverse		
OMC387	CCACTCGTGTCTGCTTTTGTGC	qPCR, hGATA6, forward	139 bp	NM_005257
OMC388	CCCTTCCCTTCCATCTTCTCTCAC	qPCR, hGATA6, reverse		
OMC429	ACCCCCTACGAGTTTACAGGTCTG	qPCR, FoxA1, forward	166 bp	BC033890
OMC430	CTGAGAAGCAAATGGCTCTGATG	qPCR, FoxA1, reverse		
OMC499	AAAGAACCCCAGCAAGGAAGAG	qPCR, hHNF1B, forward	170 bp	BC017714
OMC500	ACGGACCTCAGTGACCAAGTTG	qPCR, hHNF1B, reverse		
OMC511	TGCTGTGCCTGGGGTTTATG	qPCR,GALK1,forward	153 bp	NM_000154
OMC512	GCTGCTTGAGAGAGGTAGAAGGTG	qPCR,GALK1, reverse		
OMC513	TCACGACTTACTGGAGCAGGATG	qPCR,GALK2V1,forward	177 bp	NM_002044
OMC514	CAAAACCAAAGCCCCACCTC	qPCR,hGALK2V1, reverse		
OMC515	GGACATTTTTACACTGCTTGCCC	qPCR,hOTC, forward	105 bp	BC107153
OMC516	TCCACTTTCTGTTTTCTGCCTCTG	qPCR,hOTC, reverse		
OMC527	TGAGAAATCTGAGGCGGGAAGC	qPCR,hCYP3A4, forward	111 bp	J04449
OMC528	CGATGTTCACTCCAAATGATGTGC	qPCR,hCYP3A4, reverse		
OMC537	GTTACTTCATCCAGCCCACTGTG	qPCR,ALDH2, forward	121 bp	AY621070
OMC538	CCAACAACCTCCTCTATGGTCTTG	qPCR,hALDH2, reverse		
OMC549	GGCTGTGCTTGATGTATTTGAGG	qPCR, hA1-AT, forward	154 bp	NM_001085
OMC550	GATGTTCTGGGTGTCTGTAGGGAC	qPCR, hA1-AT, reverse		
OMC551	CTTGGCTCCTTTTGTGTTTTCCTC	qPCR, hTAT, forward	105 bp	BC020707
OMC552	GTCCAGGGCATCTTTCATTGC	qPCR, hTAT, reverse		
OMC569	AAGCACAGCGACGAGTACAA	qPCR,CEBPB, forward	155 bp	BC007538
OMC570	AGCTGCTCCACCTTCTTCTG	qPCR,CEBPB, reverse		
OMC571	AGAAGTTGGTGGAGCTGTCG	qPCR,CEBPD, forward	101 bp	BC105109
OMC572	CAGCTGCTTGAAGAACTGCC	qPCR,CEBPD, reverse		
OMC579	AACAGAGCCAGTCACAGCACCAAG	qPCR, G6P, forward	139 bp	NM_000151
OMC580	CCTCAGGAAATCCATTGATACGG	qPCR, G6P, reverse		
OMC581	GGCTACAACTTCGGCAAATACCTG	qPCR, hPEPCK, forwards	167 bp	NM_002591
OMC582	TTGAACATCCACTCCAGCACCCTG	qPCR, hPEPCK, reverse		
OMC587	AAGGAGATGCCGAAGGGGTATC	qPCR,FoxA3, forward	129 bp	BC016024
OMC588	CTGGTAGATTTCACTCAAGGTCAGC	qPCR,FoxA3, reverse		
OMC591	TGTCCATGAGCTTTCACGAG	qPCR, hPAH, forward	135 bp	NM_000277
OMC592	TTAAAACCAGGGTGGTCAGC	qPCR,h PAH, reverse		
OMC611	GCAACGGACAGATGTGTGAGTG	qPCR,hHNF4Av2, forward	146 bp	NM_000457
OMC612	AGAGAGGGGCTTGACGATTGTG	qPCR,hHMF4Av2, reverse		
OMC615	ACTCCACTCCAACCTCCAAG	qPCR,hSox7, forward	151 bp	NM_031439
OMC616	GTGGCCAGGAGTGTTCAAAT	qPCR,hSox7, reverse		
OMC631	ACCTGTCCCAACACCTCAAC	qPCR,hHNF1A, forward	152 bp	M57732
OMC632	CTCATCACCTGTGGGCTCTT	qPCR,hHNF1A, reverse		
OMC689	GATACCTCCTACTACCAGGG	qPCR,hFoxA2, forward	121 bp	NM_021784
OMC690	CACTTGCTCTCTCACTTGTC	qPCR,hFoxA2, reverse		
OMC695	TCAGTCATTTCACACCAGC	qPCR,hHNF4G, forward	126 bp	BC105011
OMC696	TGCCAAAAGTGCTATCCTG	qPCR,hHNF4G, reverse		

hepatocyte culture also presents ethical issues when cells are harvested from humans. Transplantation requires the other cell sources than primary hepatocytes.

Human induced pluripotent stem (iPS) cells have been established in culture, thus opening avenues for stem cell-based therapies [Takahashi et al., 2007]. The successful differentiation of iPS cells into hepatocytes in culture would prove useful for transplantation in patients with hepatic insufficiency. Ethical issues and graft-versus-host disease can be avoided with hiPS cells because they can be obtained from cells isolated from the intended recipient. This makes hiPS cells ideally suited as a source of cells for transplant patients.

Methods have been under investigation regarding differentiation of hiPS cells to hepatocytes. Takebe et al. engineered liver tissues using a combination of hiPS cells, human vascular endothelial cells, and human mesenchymal stem cells [Takebe et al., 2013]. Hepatocyte differentiation of hiPS cells was promoted by following an in vitro protocol [Si-Tayeb et al., 2010]. It takes several weeks to co-culture the three different cell types. 65% of patients with acute liver failure succumbed to the disease within 6 weeks after admission [Fabrega et al., 2013]. These studies indicate that quicker generation of hepatocytes is necessary for ensuring patient survival. Another problem arises when hepatocytes are produced from hiPS cells.

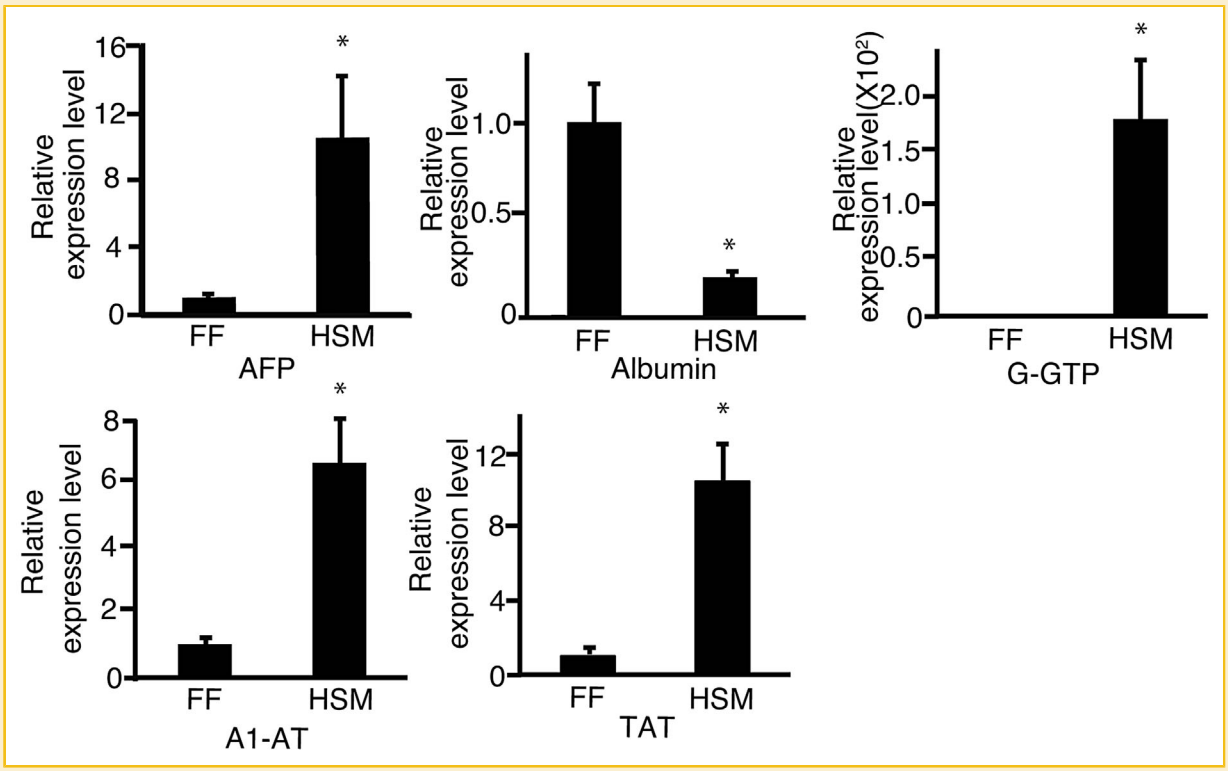

Fig. 1. Expression of liver specific genes with hepatocyte selection medium. The 201B7 cells were cultured in Repro FF or hepatocyte selection medium for 48 h. RNA was isolated and subjected to real-time quantitative PCR. Relative expression levels of liver specific genes are indicated. FF: ReproFF, HSM: hepatocyte selection medium, AFP: alpha-fetoprotein, G-GTP: γ-glutamyl transpeptidase, A1-AT: α1-anti-trypsin, TAT: tyrosine aminotransferase, error bar: standard deviation, *: $P < 0.05$ as compared with ReproFF, $n = 3$.

Residual undifferentiated cells may progress to cancer when they are mixed with hepatocytes and transplanted to patients [Okita et al., 2007].

Glucose is an important source of energy required for cell survival. Deprivation of glucose aids in the selective isolation of hepatocytes in this medium, because only they can produce this monosaccharide [Leffert and Paul, 1972]. Galactose enters glycolysis as a substrate for galactokinase, which is expressed in the liver and kidney [Ai et al., 1995; Ohira et al., 2005]. Cells cultured in vitro are highly sensitive to arginine deprivation [Wheatley et al., 2000]. Arginine is produced from ornithine by ornithine transcarbamylase (OTC) in the urea cycle, which is exclusive to hepatocytes. It is expected that cells with hepatocyte function would survive in a medium without glucose or arginine, and supplemented with galactose and ornithine. We have developed hepatocyte selection medium (HSM) that is devoid of glucose or arginine, but contains galactose and ornithine [Tomizawa et al., 2013b]. HSM enriches hepatoblast-like cells differentiated from mouse embryonic stem cells [Tomizawa et al., 2008]. Interestingly, HSM also promotes the differentiation of mouse embryonic stem cells to hepatoblast-like cells However, it is difficult to analyze hepatocyte differentiation of hiPS cells in HSM because all of the cells die within 3 days due to apoptosis in the medium [Tomizawa et al., 2013b].

Survival of hiPS cells in HSM is essential for analyzing the biological characteristics and functions of these cells. 2,2′-methylenebis (1,3-cyclohexanedione) (M50054) supplementation ensures cell survival by apoptosis inhibition induced by caspase-3 [Tsuda et al., 2001]. HSM lacks non-essential amino acids (NEAA) or pyruvate to favor the survival of hepatocyte-lineage cells over hiPS cells. NEAA is a medium supplement that has been reported to help, support, and propagate mouse embryonic stem (ES) cells [Ogawa et al., 2004]. It is added in the media used to culture 201B7 cells on a feeder layer [Takahashi et al., 2007]. Pyruvate, which is the final product of glycolysis, enters the citric acid cycle. All neural cells have been demonstrated to perish when deprived of pyruvate and glucose in the medium [Matsumoto et al., 1994]. Pyruvate is added in the medium to culture mouse ES cells [Abe et al., 1996]. HiPS cells also require NEAA and pyruvate for their survival in the culture medium because the cells cannot produce them due to the lack of enzymes required for their production. Small molecules or growth factors, including hepatocyte functional proliferation inducer (FPH1) and hepatocyte functional enhancer (FH1), promote differentiation of hiPS cells to a hepatic lineage [Shan et al., 2013]. Oncostatin M (OnM) and hepatocyte growth factor (HGF) are important for hepatocyte differentiation. OnM promotes hepatocyte differentiation at 14.5 days of gestation [Kamiya et al., 2002]. In hiPS cells, OnM has been shown to promote hepatic lineage commitment [Tomizawa et al., 2013a]. HGF has also been used for differentiation of hiPS cells to hepatocytes [Si-Tayeb et al., 2010].

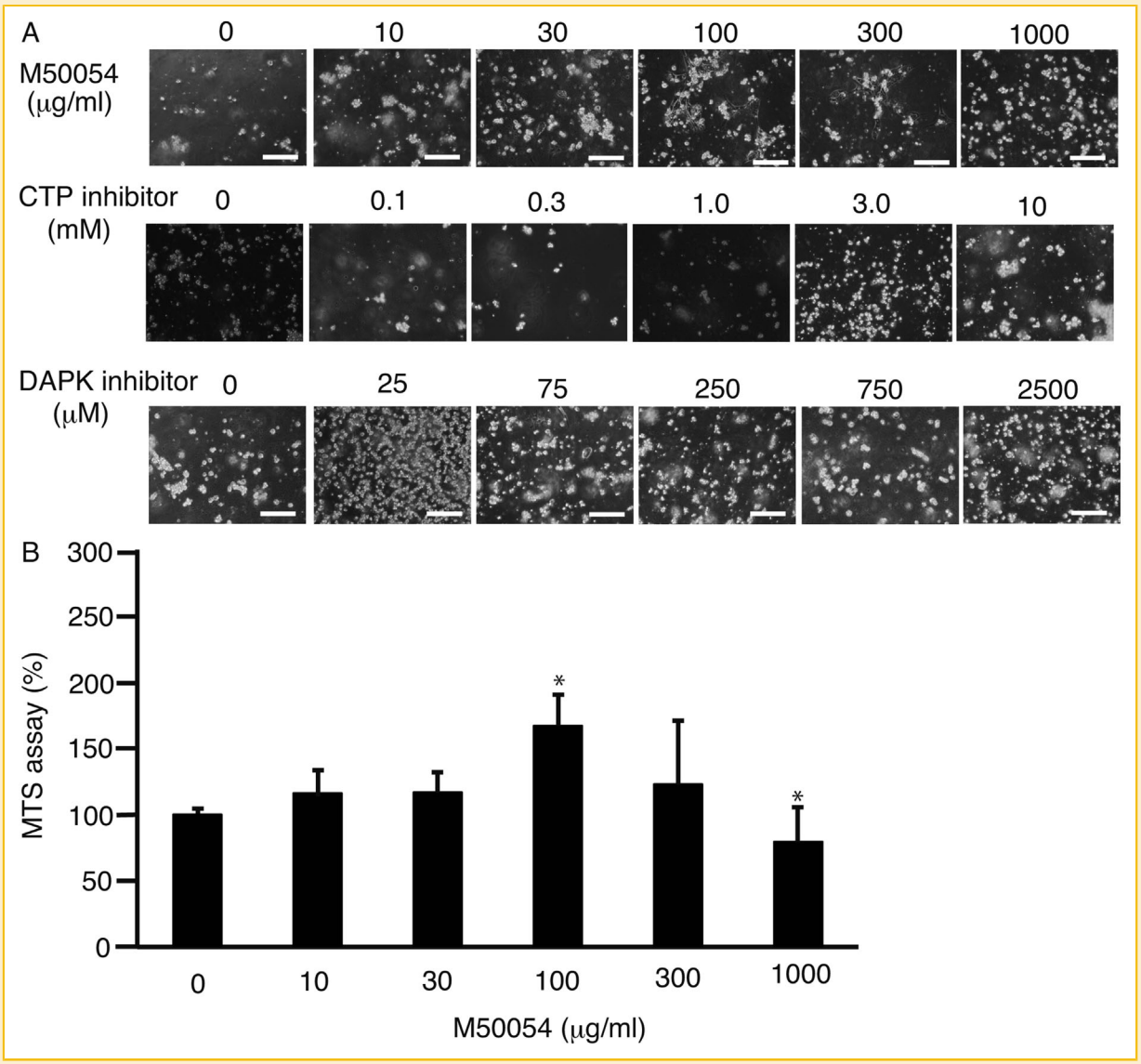

Fig. 2. Inhibitors of apoptosis in hepatocyte selection medium. The 201B7 cells were incubated with apoptosis inhibitors in hepatocyte selection medium (HSM) and observed under a microscope (A). Cell proliferation assay was performed with M50054 in HSM. CTP inhibitor: mitochondrial citrate transport protein inhibitor, DAPK inhibitor: death-associated protein kinase inhibitor, error bar: standard deviation, *: $P < 0.05$ as compared with 0 μg/ml of M50054, n = 3.

In this study, we observed an unexpected upregulation of alpha-fetoprotein (AFP) in hiPS cells after 2 days of culture in HSM. It was impossible to analyze hiPS cells beyond 3 days because they die. The cells were characterized in a medium with the combination of growth factors and small molecules in HSM in 2 days.

MATERIALS AND METHODS

CELL CULTURE

An hiPS cell line, 201B7 (Riken Cell Bank, Tsukuba, Japan), was cultured in flasks or on plates coated with Matrigel (Becton Dickinson, Franklin Lakes, NJ) in ReproFF medium (ReproCELL, Yokohama, Japan) at 37°C with 5% carbon dioxide in a humidified chamber. Once confluent, the cells were rinsed with phosphate-buffered saline and harvested with Accutase (Innovative Cell Technologies, San Diego, CA). We used 25 cm^2 flasks (Asahi Techno Glass, Funabashi, Japan) for culturing the cells and observed the cells under a microscope (CKX41N-31PHP, Olympus, Tokyo, Japan). We added growth factors in Dulbecco's Minimum Essential Medium-F12 (Sigma Aldrich, St. Louis, MO) supplemented with 10% Knockout Serum Replacement (Life Technologies, Grand Island, NY), 1× NEAA (Life Technologies), 2 mM L-Glutamine (Life Technologies), and 1 mM 2-Mercaptoethanol (Sigma–Aldrich) (iPSm[−]) to identify the growth factors that promote hepatocyte differentiation [Takahashi et al., 2007].

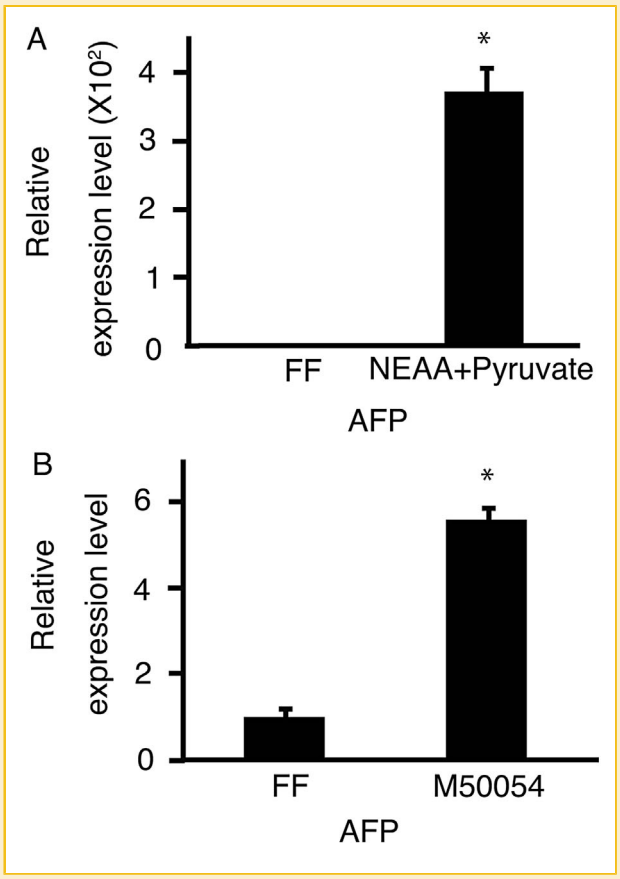

Fig. 3. Addition of non-essential amino acids and sodium pyruvate, or M50054. The 201B7 cells were cultured in hepatocyte selection medium (HSM) with non-essential amino acids and sodium pyruvate (A), or in HSM with M50054 for 48 h. Expression of alpha-fetoprotein (AFP) was analyzed by real-time quantitative PCR. FF: ReproFF, NEAA: non-essential amino acids, pyruvate: sodium pyruvate, error bar: standard deviation, *: $P < 0.05$ as compared with ReproFF, n = 3.

REAGENTS

Hundred times NEAA and 100× sodium pyruvate were purchased from Life Technologies. Inhibitors of apoptosis included the mitochondrial citrate transport protein (CTP) inhibitor ((4-Chloro-3-[[(3-nitrophenyl) amino]sulfonyl]-benzoic acid) (Sigma, St. Louis, MO), apoptosis inhibitor (M50054) (2,2′-Methylenebis(1,3-cyclohexanedione)) (Merck, Billerica, MA), and death-associated protein kinase (DAPK) inhibitor ((4*Z*)-4-(3-Pyridylmethylene)-2-styryl-oxazol-5-one) (Merck). FPH1 (2-(N-(5-chloro-2-methylphenyl) methylsulfonamido)-N-(2,6-difluorophenyl) acetamide) and FH1 (N,N′-(methylenebis(4,1-phenylene))diacetamide) were purchased from XcessBio (San Diego, CA) [Shan et al., 2013]. The growth factors were activin A (10 ng/ml) (R&D systems, Minneapolis, MN), basic fibroblast growth factor (5 ng/ml) (Wako Pure Chemicals, Osaka, Japan), β-nerve growth factor (100 ng/ml) (R&D systems), dexamethasone (10^{-7} M) (Wako Pure Chemicals), 100× insulin-transferrin-sodium-selenite media supplement (Sigma), epidermal growth factor (20 ng/ml) (Wako Pure Chemicals), transforming growth factor-β1 (2 ng/ml) (R&D systems), bone morphogenetic protein-4 (20 ng/ml) (Wako Pure Chemicals), all-trans retinoic acid (1 μM) (Sigma), OnM (20 ng/ml) (Wako Pure Chemicals), and HGF (20 ng/ml) (Wako Pure Chemicals). Nicotinamide (1.2 mg/ml), proline (30 ng/ml), and L-glutamine (0.3 mg/ml) were purchased from Wako Pure Chemicals.

HEPATOCYTE SELECTION MEDIUM AND HEPATOCYTE DIFFERENTIATION INITIATING MEDIUM

The HSM was prepared from amino acids following the formulation of Leibovits-15 medium (Life Technologies), with the omission of arginine, tyrosine, glucose, and sodium pyruvate, and the addition of galactose (900 mg/l) (Wako), ornithine (1 mM) (Wako), glycerol (5 mM) (Wako), and proline (260 mM) (Wako) [Tomizawa et al., 2013b]. Proline (30 mg/l) was added because it is necessary for DNA synthesis [Nakamura et al., 1984]. Aspartic acid was not included since it is one of the products of ornithine metabolism and a substrate for arginine synthesis. Knockout serum replacement (Life Technologies) was added at a final concentration of 10% and was used instead of FCS to establish xeno-free defined conditions. Hepatocyte differentiation initiating medium (HDI) was prepared by the addition of OnM, FPH1, M50054, 1× NEAA, 1× sodium pyruvate, nicotinamide, proline, and glutamine. Proline and nicotinamide are necessary for primary hepatocytes proliferation [Nakamura et al., 1984; Mitaka et al., 1991]. The concentration of each reagent was as mentioned above.

REAL-TIME QUANTITATIVE POLYMERASE CHAIN REACTION

201B7 cells were used for real-time quantitative polymerase chain reaction. Total RNA (5 μg), which was isolated using Isogen (Nippon Gene Co., Ltd., Tokyo, Japan), was used for the first-strand cDNA synthesis with SuperScript III reverse transcriptase and oligo (dT) primers, as per the manufacturer's instructions (Life Technologies). RNA from human fetal and adult liver was purchased from Clontech (Mountain View, CA). Real-time quantitative PCR was performed with Fast SYBR Green Master Mix (Life Technologies), and the results were analyzed using the Mini Opticon system (Bio-Rad, Hercules, CA). Real-time quantitative PCR was performed for 40 cycles, using 5 s for denaturation (95°C) and 5 s for annealing-extension (60°C) with MJ Mini Cycler (Bio-Rad). The primer sequences are presented in Table I.

CELL PROLIFERATION ASSAY

201B7 cells were trypsinized, harvested, spread onto 96-well flat-bottom plates (Asahi Techno Glass) at a density of 1,000 cells per well, and then incubated for 24 h in ReproFF medium. After culturing, cells were incubated with M50054 in HSM for 72 h. The cell cultures were subjected to 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) assays according to the manufacturer's instructions (Promega Corporation, Madison, WI). MTS is reduced biologically by cells to give a colored formazan product whose absorbance can be measured at 490 nm. Absorbance was analyzed with an iMark Microplate Absorbance Reader (Bio-Rad).

IMMUNOSTAINING

201B7 cells were spread onto 8-well chamber slides (Matsunami Glass Ind.,Ltd, Kishiwada, Japan) coated with matrigel. When the

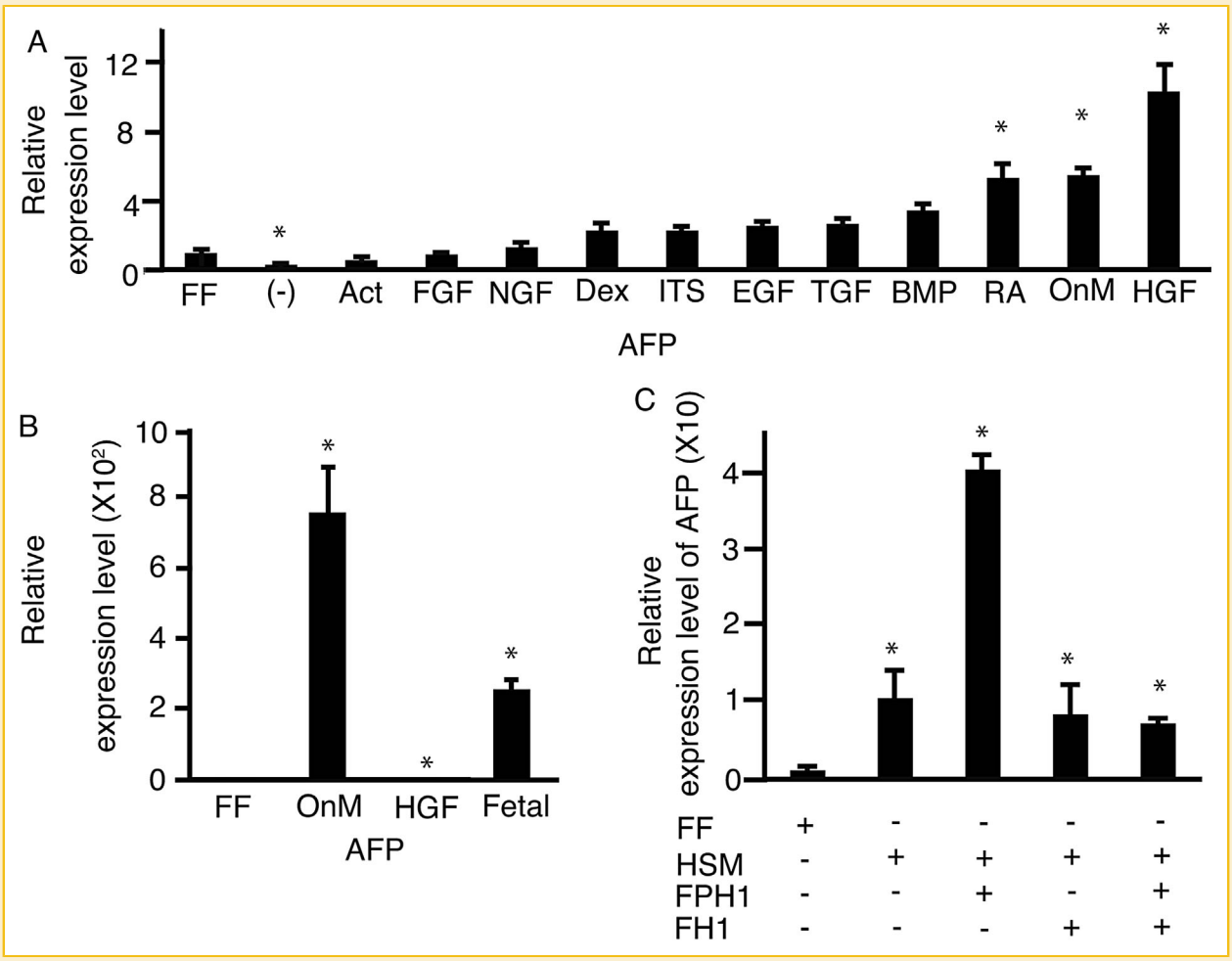

Fig. 4. Assay for growth factors that promote hepatocyte differentiation. The 201B7 cells were cultured with growth factors in iPSm (−) (see text) for 7 days. RNA was isolated, and expression of alpha-fetoprotein (AFP) was analyzed (A). The 201B7 cells were stimulated with oncostatin M or hepatocyte growth factor in HSM (B). The 201B7 cells were stimulated with small molecules that promote differentiation of human induced pluripotent stem cells (C). FF: ReproFF, (−): iPSm(−), Act: activin A (10 ng/ml), FGF: basic fibroblast growth factor (5 ng/ml), NGF: β-nerve growth factor (100 ng/ml), Dex: dexamethasone (10^{-7} M), ITS: insulin-transferrin-sodium-selenite media supplement, EGF: epidermal growth factor (20 ng/ml), TGF: Transforming growth factor-β1 (2 ng/ml), BMP: bone morphogenetic protein-4 (20 ng/ml), RA: all trans retinoic acid (1 μM), OnM: oncostatin M (20 ng/ml), HGF: hepatocyte growth factor (20 ng/ml), FPH1: hepatocyte functional proliferation inducer, FH1: hepatocyte functional enhancer, HSM: hepatocyte selection medium, fetal: fetal liver, error bar: standard deviation, *: $P < 0.05$ as compared with ReproFF, n = 3.

cells reached confluency, media were changed to HSM or HDI. After 48 h incubation as mentioned above, the cells were fixed with 4% paraformaldehyde (Sigma) for 30 min at 4°C. Internal peroxydase was inactivated with incubation 0.1% hydrogen oxide in 100% methanol for 30 min at 4°C. Specimens were incubated with 2% fetal bovine serum (Life Technologies) in phosphate buffered saline (wash buffer) for 30 min at 4°C. Specimens were incubated with 1:1000 diluted mouse anti-human AFP antibody (Takara, Otsu, Japan), in the wash buffer overnight at 4°C. Specimen were rinsed with phosphate buffered saline three times, and incubated with 1:1000 diluted horseradish peroxidase labeled anti-mouse antibody (GE Heathcare, Pittsburg, PA) for three house at 4°C. Diaminobenzidine (DAKO, Glostrup, Denmark) was applied, and the nuclei were stained with hematoxylin (Muto Pure Chemicals, Tokyo, Japan) for 15 s. Specimens were observed and photographed with AX80 (Olympus, Tokyo, Japan).

STATISTICAL ANALYSIS

One-way analysis of variance (ANOVA) was applied with JMP 10.0.2 software (SAS Institute, Cary, NC). A P value <0.05 was employed statistically significant. The Tukey–Kramer analysis method was used to search for pairs of data with statistical significance.

RESULTS

RNA was isolated from 201B7 cells and subjected to real-time quantitative PCR after 2 days of culture in HSM (Fig. 1). Unexpectedly, AFP levels were upregulated about 10-fold (10.3 ± 1.9; mean ±standard deviation) in the 201B7 cells cultured with HSM than in those cultured with ReproFF medium. This suggested that 201B7 cells had started to differentiate to the hepatocyte lineage.

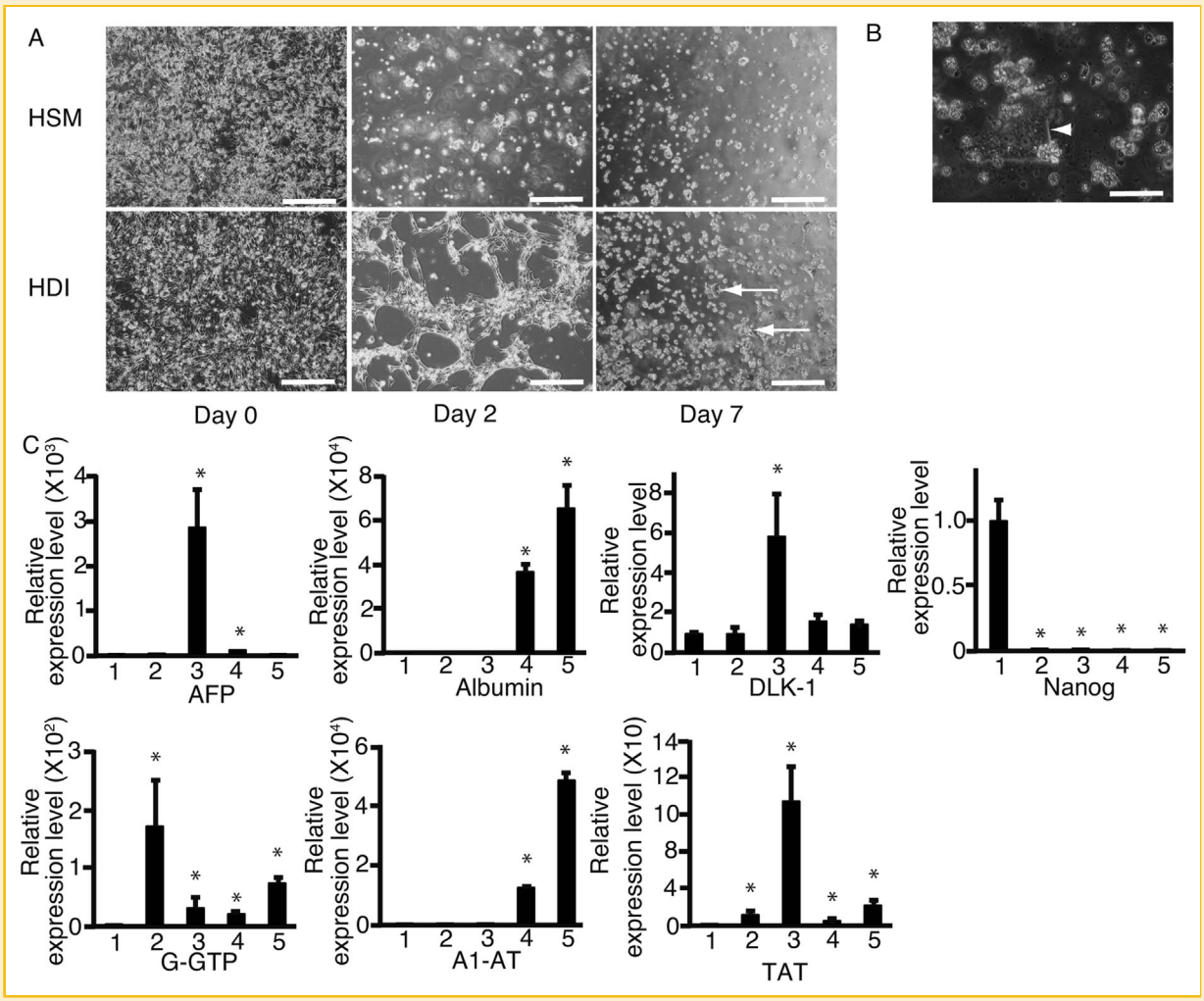

Fig. 5. Expression of hepatocyte-specific genes. Hepatocyte selection medium (HSM) was prepared with the omission of arginine and glucose, and the addition of galactose and ornithine (see text). Hepatocyte differentiation inducer (HDI) was prepared with the addition of with non-essential amino acids, sodium pyruvate, M50054, hepatocyte functional proliferation inducer, hepatocyte functional enhancer, and oncostatin M in HSM (see text). The 201B7 cells were cultured in HSM or HDI for 7 days (A). Small number of cells survived in HDI for 7 days (arrows). Binucleated cells were found in the cells cultured in hepatocyte differentiation inducer for 7 days (arrowhead) (B). RNA was isolated and subjected to real-time quantitative polymerase chain reaction to analyze the expression levels of alpha-fetoprotein (AFP), albumin, delta-like-1 (DLK-1), Nanog, γ-glutamyl transpeptidase (G-GTP), α1-anti-trypsin (A1-AT), and tyrosine aminotransferase (TAT) (C). Lane 1: ReproFF, 2: hepatocyte selection medium, 3:hepatocyte differentiation inducer, 4: fetal liver, 5: adult liver, arrows: surviving cells, arrowhead: a binucleated cell, original magnification: 100×, scale bar: 50 μm, error bar: standard deviation, *: $P < 0.05$ as compared with ReproFF, n = 3.

The 201B7 cells have been reported to die after 3 days of culture in HSM due to apoptosis [Tomizawa et al., 2013b]. We added apoptosis inhibitors in the medium to facilitate the survival of 201B7 cells. M50054, CTP inhibitor, or DAPK inhibitor were added in HSM and survival of 201B7 cells was verified by microscopic observation (Fig. 2A). After 3 days in culture, no cells survived in the presence of CTP inhibitor or DAPK inhibitor. However, the 201B7 cells survived in the media with M50054 at a concentration of 30, 100, and 300 μg/ml. It appeared that cell survival was maximal at a concentration of 100 μg/ml of M50054. To confirm this, the MTS assay was performed (Fig. 2 B). Cell proliferation was estimated to have increased to 167% ± 19.9% in cells cultured with 100 μg/ml of M50054 than in the controls cultured in the media lacking M50054 ($P < 0.05$).

NEAA and pyruvate were thought to be necessary for the 201B7 cells to survive. AFP expression was upregulated to 371% ± 33.9% after 2 days of culture with NEAA and pyruvate in HSM than cultures with ReproFF medium (Fig. 3A). The surviving 201B7 cells, after 2 days of culture with M50054 in HSM, showed a 5.6 ± 0.3 fold increase in expression of AFP (Fig. 3 B).

The growth factors were screened for efficient promotion of differentiation of the 201B7 cells to a hepatic lineage. The 201B7 cells were cultured for 7 days with each growth factor in iPSm(−) (Fig. 4A). The use of HGF and OnM in the medium resulted in the highest increase in the expression of AFP. We then cultured the 201B7 cells for 2 days with either OnM or HGF in HSM, and analyzed AFP expression (Fig. 4B). AFP expression was found to be upregulated with OnM. We also found that FPH1 and FH1, small

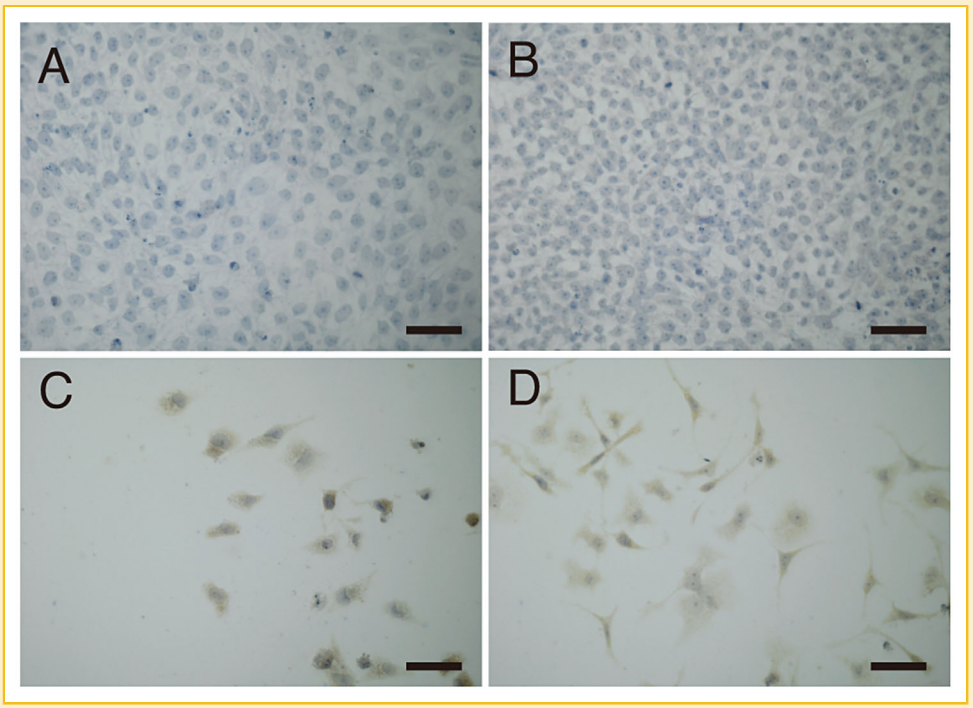

Fig. 6. Immunostaining. 201B7 cells were incubated with Repro FF (A, B), hepatocyte selection medium (C), or hepatocyte differentiation inducer (D) for 48 h, and subjected to immunostaining without antibody to human alpha-feto protein (A) or with the antibody (B, C, D). Original magnification: ×200, scale bar: 50 μm.

molecules, promote hepatocyte differentiation. The 201B7 cells were cultured in HSM supplemented either one or with a combination of the small molecules, and the expression of AFP was analyzed (Fig. 4C). FPH1 expression was solely identified to upregulated AFP.

We used the combination of M50054, NEAA, pyruvate, and OnM in HSM to prepare the Hepatocyte differentiation initiating medium (HDI). The 201B7 cells were cultured in HSM or HDI for 7 days (Fig. 5A). Most of the cells died in HSM in 2 days. Part of the cells survived in HDI for 2 days. After 7 days, none of the cells survived in HSM. Small number of cells survived in HDI. A binucleated cell was found among cells cultured in HDI for 7 days (Fig. 5B). This morphological feature suggested that the surviving cells acquired the characteristics of cells differentiating toward hepatocyte-lineage cells. Expressions of AFP and γ-glutamyl transpeptidase (γ-GTP) were also upregulated in the cells cultured with HDI, suggesting that the 201B7 cells had differentiated into hepatoblast-like cells (Fig. 5C). Interestingly, the expression of delta-like (DLK) 1, another marker of hepatoblasts, was also upregulated. Expression of Nanog disappeared, suggesting that the cells in HDI had lost pluripotency. Similarly, albumin and α1-antitrypsin were also not expressed.

To analyze the expression level of AFP at protein level, 201B7 cells were incubated with hepatocyte differentiation inducer for 48 h, and subjected to immunostaining with antibody to human alpha-feto protein. The cells were negative without primary antibody (Fig. 6A). The cells incubated with ReproFF were negative for AFP (Fig. 6B). The cells incubated with HSM (Fig. 6C) or HDI (Fig. 6 D) were positive for AFP. It was confirmed that AFP was expressed at protein level.

Expression levels of transcription factors specific to hepatocytes were analyzed with the 201B7 cells cultured in HDI for 2 days (Fig. 7). The expression of all the transcription factors was found to be increased to levels comparable to their levels in fetal liver.

The expression levels of genes involved in metabolism were analyzed from the 201B7 cells cultured in HDI for 2 days (Fig. 8). Expressions of GALK2 and OTC were upregulated. These results suggested that galactose metabolism and urea cycle were activated. This was anticipated, because hepatoblast-like cells had to meet the need for glucose and arginine. The expression levels of genes involved in drug metabolism were also analyzed. CYP3A4 was found to be upregulated to a level comparable to that in fetal liver. The expression of ALDH, however, was weak.

DISCUSSION

One major question was what type of cells were obtained with HDI. Bi-nucleated cells were found after 7 days culture in HDI (Fig. 6B). It was suggested that the 201B7 cells were differentiating toward hepatocye-lineage because a bi-nucleated cell was a morphological character of hepatocyte-lineage cells [Tomizawa et al., 2008]. The cells that survived the culture with HDI expressed panels of liver-specific genes. The 201B7 cells expressed AFP and γ-GTP. The former is a marker of immature hepatocytes and the latter, of bile duct epithelial cells [Tateno et al., 2010; Inamura et al., 2011]. These results suggested that the cells were hepatoblast-like cells because they had characters of both immature hepatocytes and bile duct

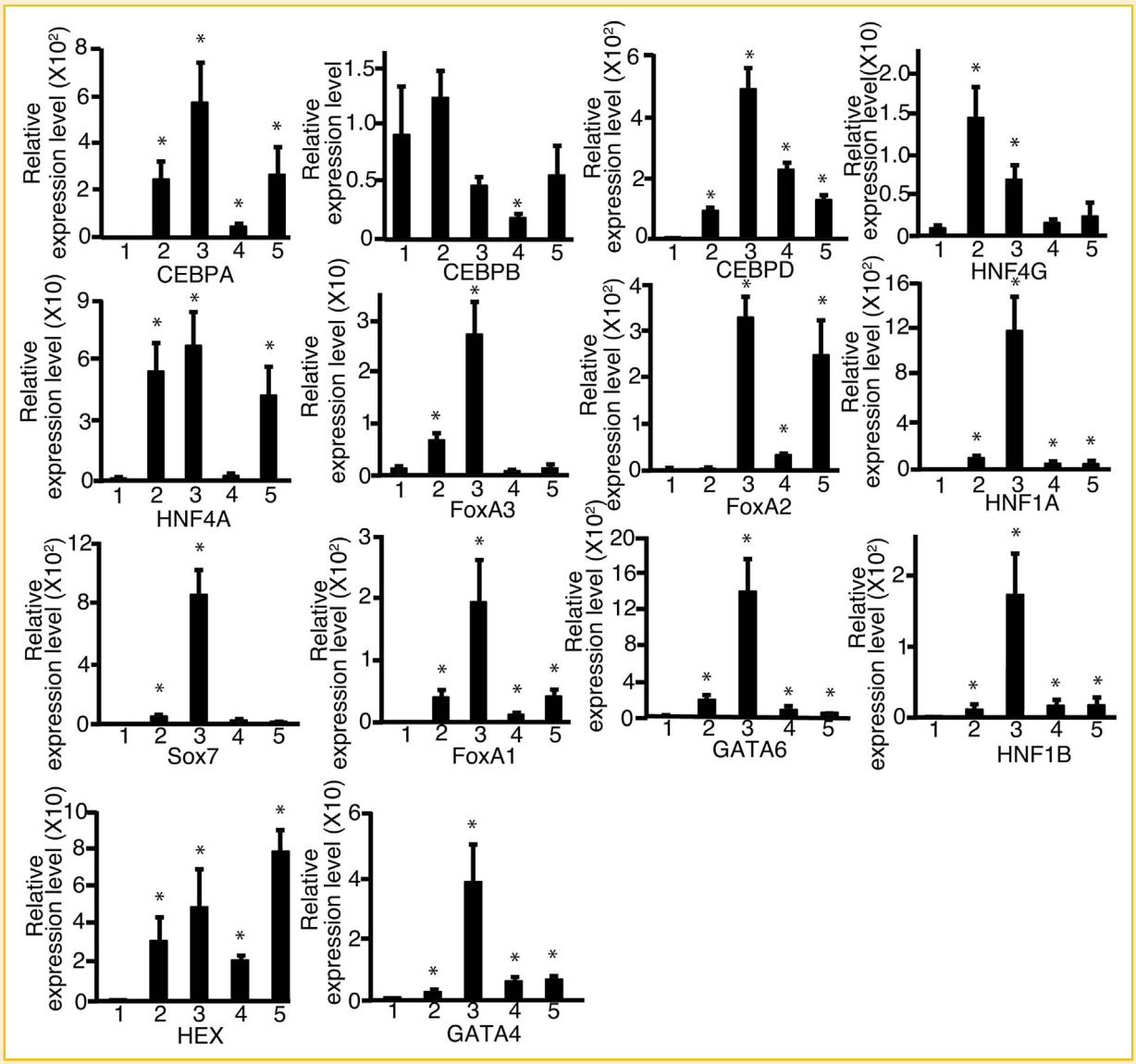

Fig. 7. Expression of hepatocyte-specific transcription factors. The 201B7 cells were stimulated with non-essential amino acids, sodium pyruvate, M50054, hepatocyte functional proliferation inducer, hepatocyte functional enhancer, and oncostatin M (hepatocyte differentiation inducer) for 2 days. RNA was isolated and subjected to real-time quantitative polymerase chain reaction to analyze the expression levels of CCAAT/enhancer binding protein α (CEBPA), CCAAT/enhancer binding protein β (CEBPB), CCAAT/enhancer binding protein δ (CEBPD), hepatocyte nuclear factor 4γ (HNF4G), hepatocyte nuclear factor 4α transcript variant 2 (HNF4A), forkhead box A3 (FoxA3), forkhead box A2 (FoxA2), hepatocyte nuclear factor 1α (HNF1A), sex determining region Y-box7 (Sox7), forkhead box A1 (FoxA1), GATA binding protein 6 (GATA6), hepatocyte nuclear factor 1β (HNF1B), hematopoietically expressed homeobox (HEX), and GATA binding protein 4 (GATA4). Lane 1: Repro FF, 2: hepatocyte selection medium, 3: hepatocyte differentiation inducer, 4: fetal liver, 5: adult liver, error bar: istandard deviation, *: $P < 0.05$ as compared with ReproFF, n = 3.

epithelial cells [Tomizawa et al., 1998]. This result was supported by the expression of DLK-1, which is expressed in hepatoblasts [Tanaka et al., 2009].

Our results suggested that hepatocyte differentiation was initiated in HDI when the 201B7 cells expressed GALK2 and OTC to survive. Hepatocyte differentiation might be triggered by an unknown mechanism resulting in expression of GALK2 and OTC. Our speculation was that hiPS cells attempted to differentiated to hepatoblast-like cells to obtain glycolysis and the urea cycle to survive HDI. Once hiPS cells obtained glycolysis and the urea cycle, they could survive HDI metabolizing galactose and ornithine to glucose and arginine, respectively.

On the other hand, expression of albumin and α1-antitrypsin was not observed. The surviving cells in HDI did not fully gain functions specific to hepatocytes. This speculation might be the reason why small number of cells survived in HDI for 7 days.

Figure 5 showed that the expression patterns of iPS cells cultured in HSM and HDI were different. γ-GTP was more up-regulated in HSM than HDI. AFP and DLK-1 were more up-regulated in HDI than HSM. γ-GTP is a marker of bile duct

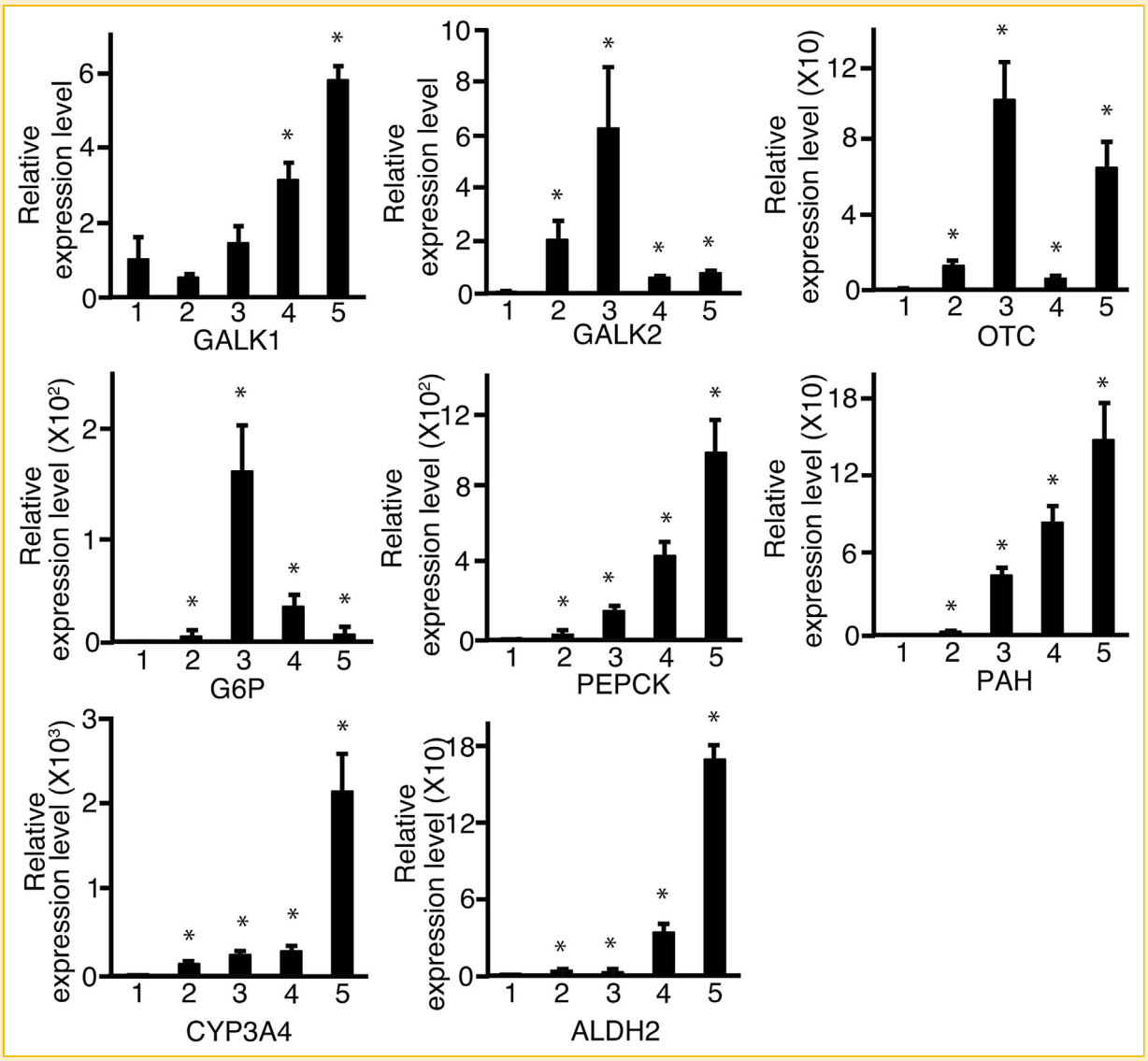

Fig. 8. Expression levels of metabolic genes. The 201B7 cells were cultured with non-essential amino acids, sodium pyruvate, M50054, hepatocyte functional proliferation inducer, hepatocyte functional enhancer, and oncostatin M (hepatocyte differentiation inducer) for 2 days. RNA was isolated and subjected to real-time quantitative polymerase chain reaction to analyze the expression of galactokinase-1 (GALK1), galactokinase-2 (GALK2), ornithine transcarbamylase (OTC), glucose-6-phosphatase (G6P), phosphoenolpyruvate carboxykinase (PEPCK), and phenylalanine hydroxylase (PAH), cytochrome P-450 nifedipine oxidase (CYP3A4), and aldehyde dehydrogenase 2 (ALDH). Lane 1: ReproFF, 2: hepatocyte selection medium, 3: hepatoctye differentiation inducer, 4: fetal liver, 5: adult liver, error bar: standard deviation, *: $P < 0.05$ as compared with ReproFF, $n = 3$.

epithelial cells. AFP and DLK-1 were markers of hepatoblasts. Our results suggested that HSM and HDI might promote the differentiation of iPS cells to bile duct epithelial cells and hepatoblasts, respectively. These results suggested that direction of differentiation of iPS cells might be different between HSM and HDI. HSM promotes the differentiation of mouse ES cells to hepatoblast-like cells [Tomizawa et al., 2008]. It would be speculated that differentiation with HSM might differ between mouse ES cells and human iPS cells. HDI was more suitable for the differentiation of human iPS cells to hepatoblast-like cells.

Limitations of HDI were that small numbers of cells were obtained in HDI, and that obtained cells were hepatoblat-like cells not mature hepatocytes. These might arise from the same reason that the surviving cells did not acquire full functions of mature hepatocytes.

Growth factors may be involved in tumorigenicity. HGF promotes proliferation of hepatocellular carcinoma and an inhibitor of its receptor suppresses cell proliferation [Tomizawa et al., 2014]. OnM may be involved in cervical cancer and a target of a therapy [Caffarel and Coleman, 2014]. HDI did not include HGF but OnM. The 201B7 cells might be transformed to cancer cells cultured in HDI. Most of the cells died in 2 days in HSM. It might be possible to eliminate cancer cells transformed from iPS cells with HDI. In the future, hepatocellular carcinoma cells would be subjected to culture in HSM, and their survival would be observed.

In conclusion, differentiation of 201B7 cells to hepatoblast-like cells was initiated in HSM supplemented with non-essential amino acid, sodium pyruvate, M50054, FPH1, and OnM.

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