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Prediction of Differentiation Tendency Toward Hepatocytes from Gene Expression in Undifferentiated Human Pluripotent Stem Cells

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Abstract

Functional hepatocytes derived from human pluripotent stem cells (hPSCs) have potential as tools for predicting drug-induced hepatotoxicity in the early phases of drug development. However, the propensity of hPSC lines to differentiate into specific lineages is reported to differ. The ability to predict low propensity of hPSCs to differentiate into hepatocytes would facilitate the selection of useful hPSC clones and substantially accelerate development of hPSC-derived hepatocytes for pharmaceutical research. In this study, we compared the expression of genes associated with hepatic differentiation in five hPSC lines including human ES cell line, H9, which is known to differentiate into hepatocytes, and an hPSC line reported with a poor propensity for hepatic differentiation. Genes distinguishing between undifferentiated hPSCs, hPSC-derived hepatoblast-like differentiated cells, and primary human hepatocytes were drawn by two-way cluster analysis. The order of expression levels of genes in undifferentiated hPSCs was compared with that in hPSC-derived hepatoblast-like cells. Three genes were selected as predictors of low propensity for hepatic differentiation. Expression of these genes was investigated in 23 hPSC clones. Review of representative cells by induction of hepatic differentiation suggested that low prediction scores were linked with low hepatic differentiation. Thus, our model using gene expression ranking and bioinformatic analysis could reasonably predict poor differentiation propensity of hPSC lines.

Keywords: human embryonic stem cells, human induced pluripotent stem cells, cell differentiation, endoderm, hepatocyte

Introduction

Human pluripotent stem cells (hPSCs), including embryonic stem cells (hESCs) [1] and human induced pluripotent stem cells (hiPSCs) [2,3], are expected to be a promising cell reagents for basic biological research, cell-based medicine, and pharmaceutical development. It is because of their abilities to replicate indefinitely and to differentiate into most cell types in the body [4–6]. In pharmaceutical research, primary human hepatocytes are used as in vitro models to assess metabolism and toxicity of drug compounds. However, primary human hepatocytes cannot be passaged, rapidly lose their function [7], and are expensive. Functional hepatocytes derived from hPSCs have great potential as tools for modeling diseases [8,9], hepatitis infection [10–12], drug metabolism [13–18], fluid shear stress [19], and regeneration regenerative medicine [20]. Several protocols for hepatic differentiation of hPSCs have been reported

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[13,21–33]. Previously the Mizuguchi lab developed efficient methods to differentiate hPSCs into functional hepatocyte-like cells using adenovirus vector-mediated overexpression of hepatocyte linage-related transcription factors [34–39]. Because adenovirus vectors are the most efficient among gene delivery vehicles that can provide high transduction efficiency in both dividing and nondividing cells, the differentiation into hepatocytes should be relatively stable [40]. However, expression levels of metabolic enzymes vary between hPSC-derived hepatocyte-like cells that are derived from even a donor cell line [35,38]. Previous studies demonstrated that individual hPSC lines have distinct propensities to differentiate into specific lineages [41–45]. hESCs generated from various donors by different methods display phenotypic variation. Variation in hiPSC clones even arise from a single donor. Gene expression profiles revealed that phenotypes of hepatocytes derived from hPSCs using differentiation protocols reported until now are still immature [32,46]. Selection of good hPSC lines or clones by identifying clones exhibiting low propensity to differentiate into hepatocytes would substantially accelerate application of hPSC-derived hepatocytes in pharmaceutical research. Several studies demonstrated that variation in hepatic differentiation can be attributed either to differences between donors [47] or epigenetic memory [48,49]. Hepatic differentiation propensity was recently reported to be affected by alterations in nuclear receptor genes [50].

We hypothesized that variations in hepatocyte differentiation efficiency can be predicted by studying the gene expression profile of undifferentiated hPSCs. Although DNA arrays can provide fundamental information, analysis of the resulting data is very time-, labor-, and cost- intensive. In this study gene expression of the following five representative hPSC lines was compared using polymerase chain reaction (PCR) arrays and bioinformatic analysis: H9, which is known to differentiate well [24,29–33,35–37,39,46]; Tic and Dotcom, both of which were reported to differentiate well [34,35,37]; Squeaky, which was derived from the same donor cell line as Tic and Dotcom; and 201B7, which was reported to have a poor propensity for hepatic differentiation [38,47]. We particularly focused on genes encoding hepatocyte linage markers and growth factors involved in differentiation, and endoderm differentiation signaling pathways including PI3K-AKT signaling, WNT signaling, and TGFβ/BMP signaling. This analysis suggested three candidate genes, the low expression of which may be associated with low propensity for hepatic differentiation. We then examined expression patterns in 23 hPSC clones to estimate their hepatic differentiation potential. Using this approach based on gene expression ranking and bioinformatic analysis, we could reasonably predict lower propensity for hepatic differentiation.

Materials and Methods

Ethics statement

hESCs were used following the ''Guidelines for Utilization of Human Embryonic Stem Cells'' of the Ministry of Education, Culture, Sports, Science and Technology of Japan after approval by the Institutional Ethical Review Board of the National Institutes of Biomedical Innovation, Health, and Nutrition. Experiments using human iPSCs were conducted with the approval of the Institutional Review Board of the National Institutes of Biomedical Innovation, Health, and Nutrition.

Cell culture

Eight hESC lines: H9 (WA09), HES3, HES4, KhES-1, KhES-2, KhES-3, KhES-4, and KhES-5; and 13 hiPSC lines: 201B7, 201B6, 201B2, 253G1B1, iPS-TIG108-4f3, iPS-TIG114-4f1 (JCRB1437), iPS-TIG120-4f1 (JCRB1363), Tic (JCRB1331), Dotcom (JCRB1327), Squeaky (JCRB1329), Toe (JCRB1338), UTA1, iPS-DF19-9-7T, and its karyotypically aberrant subclone (19-9-7Tv) were used in this study. Detailed information about these cell lines and their maintaining culture conditions is provided in Supplementary Table S1 and Supplementary Materials and Methods (Supplementary Data are available online at www.liebertpub.com/scd). In brief, hPSCs were routinely maintained on inactivated mouse embryonic fibroblast feeder cells (Merck Millipore, Darmstadt, Germany) in knockout serum replacement (KSR)-based medium supplemented with 4–10 ng/mL human recombinant FGF-2 (Katayama Chemical Industries Co., Ltd., Osaka, Japan) as previously described [3,51–53]. H9, Tic, 201B7, 201B6, and 253G1B1 were cultured in an animal product-free defined medium, hESF-FX (PCT/JP2011/004691)[54] modified from a growth factor defined culture medium, hESF9, which we previously developed for culturing hESCs [55], without feeder cells on 2 μg/cm^2 bovine fibronectin [34,53,55–58] (Supplementary Data). hiPSCs, iPS-DF19-9-7T and iPS-DF19-9-7Tv were cultured in mTeSR1 on Matrigel™ according to the manufacturer's instructions (STEMCELL Technologies, Inc., Vancouver, Canada).

Primary human hepatocyte culture

Cryopreserved human primary hepatocytes (VERITAS) were cultured according to the manufacturer's instructions. The hepatocytes were seeded at 1.25×10^5 cells/cm^2 in hepatocyte culture medium (HCM; Lonza, Walkersville, MD) containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific) onto type I collagen (Nitta Gelatin)-coated plates. The medium was replaced at 6 h after seeding. The hepatocytes, which were cultured 48 h after plating the cells, were used in the experiments.

Differentiation of hPSCs into hepatoblast-like cells

hPSCs were differentiated toward hepatoblast-like cells according to a previously reported differentiation protocol [30,59] with minor modifications. In brief, hPSCs were dissociated into single cells using Accutase (Merck Millipore) and then plated at 600,000 cells per well in 24-well plates (BD Falcon, Franklin Lakes, NJ) precoated with 4 μg/cm^2 bovine fibronectin (Sigma-Aldrich, St. Louis, MO). The cells were cultured with chemically defined medium (CDM) consisting of 50% IMDM (Thermo Fisher Scientific) plus 50% F-12 Nutrient Mixture (Thermo Fisher Scientific), 7 μg/mL insulin (Roche Diagnostics), 15 μg/mL transferrin (Roche Diagnostics), 450 μM monothioglycerol (Sigma-Aldrich), and 5 mg/mL bovine serum albumin fraction V (Wako Pure Chemical Industries, Ltd., Osaka, Japan), supplemented with 10 ng/mL activin A (R&D Systems, MN) and 12 ng/mL FGF-2 for 2 days. For the following 3 days,

cells were cultured with 10 μM Ly294002 (Cell Signaling Technology, Beverly, MA), 100 ng/mL activin A, 20 ng/mL FGF-2, and 10 ng/mL BMP-4 (R&D Systems). The cells were cultured for 3 days in the presence of 50 ng/mL FGF-10 (R&D Systems) and then treated with 0.1 μM retinoic acid (Sigma-Aldrich), 10 μM SB431542 (Tocris Bioscience, Bristol, United Kingdom), and 50 ng/mL FGF-10 for 2 days. Finally, the cells were cultured in CDM supplemented with 30 ng/mL FGF-4 (R&D Systems), 50 ng/mL hepatocyte growth factor (HGF; R&D Systems), and 50 ng/mL epidermal growth factor (EGF; R&D Systems) for an additional 20 days.

Differentiation of hPSCs into hepatocyte-like cells

To confirm hepatic differentiation tendency of hPSC H9, KhES4, KhES3, and 201B7 maintained under KSR-based culture conditions, and 19-9-7T and its aberrant clone maintained under mTeSR1 culture conditions were differentiated toward hepatocyte-like cells according to previously reported differentiation protocol [31,60–62] with minor modifications. Briefly, dissociated hPSCs were cultured onto Matrigel Basement Membrane Matrix Growth Factor Reduced (Corning) in the MEF-conditioned medium for 3–4 days. Then, the cells were cultured for 4 days with the L-WNT3A-expressing cell (CRL2647; ATCC)-conditioned RPMI1640 medium (Sigma) containing 100 ng/mL Activin A (R&D Systems), 1% GlutaMAX (Thermo Fisher Scientific), 0.2% FBS, and 1×B27 Supplement Minus Vitamin A (Thermo Fisher Scientific). The hPSC-derived definitive endoderm cells were cultured with RPMI1640 medium containing 30 ng/mL bone morphogenetic protein 4 (BMP4; R&D Systems) and 20 ng/mL FGF-4 (R&D Systems), 1% GlutaMAX, and 1×B27 Supplement Minus Vitamin A for 5 days. Next, the hPSC-derived hepatoblasts were cultured with RPMI1640 medium containing 20 ng/mL HGF (R&D Systems), 1% GlutaMAX, and 1×B27 Supplement Minus Vitamin A for 5 days. Finally, the cells were cultured with the hepatic maturation medium (hepatic maturation medium consists of HCM (Lonza, without EGF) containing 20 ng/mL oncostatin M (OsM) and 3% GlutaMAX) for 11 days.

To confirm hepatic differentiation tendency of hPSC, H9, 253G1B1, and 201B7 after culturing in hESF-FX medium for more than five passages, the hPSCs were differentiated toward hepatocyte-like cells according to another previously reported differentiation protocol [31,60,61] with minor modifications. In brief, hPSCs dissociated with Accutase were seeded at a density of 600,000 cells per well in 24-well plates precoated with 300 μL/well Geltrex (Thermo Fisher Scientific) and cultured with mTeSR1 medium (Stemcell Technologies, Vancouver, Canada) in 4% O_2/5% CO_2. Approximately 24 h after seeding, when the cells were 85%–95% confluent, the medium was changed to RPMI1640 medium (Thermo Fisher Scientific) supplemented with B27 minus insulin, 100 ng/mL activin A, 10 ng/mL BMP-4, and 20 ng/mL FGF-2 in ambient O_2/5% CO_2 for 2 days. The cells were cultured in RPMI1640 medium supplemented with B27 minus insulin and 100 ng/mL activin A for the following 3 days in ambient O_2/5% CO_2. The medium was changed daily. Cells were cultured in RPMI1640 supplemented with B27 supplement (Thermo Fisher Scientific), 20 ng/mL BMP-4, and 10 ng/mL FGF-2 for 5 days in 4% O_2/5% CO_2. The medium was changed daily. Then, the medium was replaced with RPMI1640 supplemented with B27 supplement and 20 ng/mL HGF, and the cells were cultured for 5 days in 4% O_2/5% CO_2. Finally, the medium was replaced with Hepatocyte Culture Media (Lonza, Basel, Switzerland) containing SingleQuots omitting the EGF with 20 ng/mL Oncostatin-M (R&D Systems), and the cells were cultured for 5 days in ambient O_2/5% CO_2.

Albumin secretion

During the final differentiation step described above, cell culture supernatant of the hPSC-derived hepatocyte-like cells was collected every 24 h to measure albumin secretion. The primary human hepatocytes were cultured for 24 h and their culture supernatant was collected to measure albumin secretion. The human albumin content of the supernatant was determined using a Human Albumin ELISA Quantitation kit, according to the manufacturer's instructions (Bethyl Laboratories, Montgomery, TX). Albumin secretion was normalized to the total RNA of the cells from which culture supernatant was collected.

Urea production

The culture supernatants, which were incubated for 24 h after fresh medium was added, were collected and analyzed for the amount of urea production. Urea measurement kits were purchased from BioAssay Systems. The experiment was performed according to the manufacturer's instructions. The amount of urea secretion was calculated according to each standard followed by normalization to the RNA content.

CYP3A4 activity

To measure the cytochrome P450 3A4 activity of the hepatocyte-like cells and primary hepatocytes, we performed lytic assays by using a P450-GloTM 3A4 (catalog number; V9001) Assay Kit (Promega). We measured the fluorescence activity with a luminometer (Lumat LB 9507; Berthold) according to the manufacturer's instructions. The CYP activity was normalized with the RNA content.

Immunocytochemical staining

Immunocytochemistry was performed as described previously [56,57]. The primary and secondary antibodies used in this study are listed in Supplementary Table S2. Image analysis was performed using In Cell analyzer 2000 and Developer Toolbox software (GE Healthcare UK Ltd., Little Chalfont, United Kingdom).

Gene expression analysis

Total RNA was isolated from hPSCs, hepatoblast-like cells, hepatocyte-like cells, HepG2 cells, and adult hepatocytes using the RNeasy Mini Kit or RNeasy Micro Kit (Qiagen, Valencia, CA) and treated with DNase I (Qiagen) to remove any genomic contamination. For real-time PCR, 500 ng RNA was used for reverse transcription with Superscript VILO cDNA Synthesis Kit (Thermo Fisher

Scientific). PCR was performed based on the TaqMan or SYBR green gene expression technology in a 7300 Real-Time PCR System (Thermo Fisher Scientific), following the manufacturer's instructions. Threshold cycles were normalized to the housekeeping genes *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), *beta-2-microglobin* (*B2M*), and *hypoxanthine phosphoribosyltransferase 1* (*HPRT1*) and translated to relative values. Specific primers used are listed in Supplementary Table S3.

For PCR array, 500 ng of RNA of the cells and total RNA of human fetal and adult liver (Clontech Laboratories, Takara Bio USA, Inc., Mountain View, CA) was used for reverse transcription with RT^2 First Strand Kit (Qiagen) or High Capacity RNA-to-cDNA kit (Thermo Fisher Scientific) for RT^2 Profiler™ PCR Array or TaqMan Array, respectively. The expression of genes was determined using RT^2 Profiler™ PCR Array with RT^2 SYBR Green/ROX qPCR Master Mix (Qiagen) or using TaqMan Array with an ABI7900 HTR-PF (Thermo Fisher Scientific). The PCR Arrays used in this study included Human PI3K-AKT Signaling Pathway RT^2 Profiler PCR Array (87 genes), Human TGFβ/BMP Signaling Pathway RT^2 Profiler PCR Array (87 genes), Human Growth Factors RT^2 Profiler PCR Array (87 genes), Human WNT Signaling Pathway RT^2 Profiler PCR Array (87 genes), and Custom PCR array (122 genes related to hepatic differentiation and six endogenous control genes; Qiagen), described in Supplementary Table S4. Expression of stem cell markers and three germ markers was investigated using TaqMan Human Stem Cell Pluripotency Array (92 genes and 6 endogenous control genes, Thermo Fisher Scientific) with TaqMan Gene Expression Master Mix (Thermo Fisher Scientific) and ABI7900HT Fast Real-Time PCR systems (Thermo Fisher Scientific) as previously described [41]. Expression levels of all target genes were normalized to housekeeping genes *GAPDH*, *B2M*, and *HPRT1*. All data analysis was followed by bioinformatics analysis.

Bioinformatics analysis

The bioinformatics analysis flow is summarized in Supplementary Fig. S1. Data were analyzed using DataAssist™ Software v3.01 (Thermo Fisher Scientific) for RT^2 Profiler™ PCR Array (Human PI3K-AKT Signaling Pathway, Human TGFβ/BMP Signaling Pathway, and Human Growth Factors, Human WNT Signaling Pathway) and Custom PCR array. Data were also analyzed using EspressionSuite v1.0.3 (Thermo Fisher Scientific) for TaqMan Human Stem Cell Pluripotency Array. For clustering analysis, normalization of ΔC_t values was based on three reference genes (*GAPDH*, *B2M*, and *HPRT1*) in RT^2 Profiler™ PCR Array and Custom array and three reference genes (*18S ribosomal RNA*, *beta-actin*, and *GAPDH*) in TaqMan Human Stem Cell Pluripotency Array. Both analyses were completed following standard protocols. Normalization of relative quantification was based on 201B7. Median fold change was calculated as a score from each gene category [63,64]. For example, the score for endoderm in H9 (1.08) was a median fold change of 18 for endoderm marker genes. For hierarchical clustering analysis (average linkage), Cluster 3.0 v1.50 (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm) was used and for visualization, Java TreeView v1.1.6r2 (http://jtreeview.sourceforge.net) was used.

Results

Differentiation of hepatic lineage in five hPSC lines

We computed an average gene expression score for the propensity for hepatic differentiation of the five hPSC lines H9, which is known to differentiate well [24,29–33,35–37,39,46]; Tic and Dotcom, both of which are reported to differentiate well [34,35,37]; Squeaky, which was derived from the same donor cell line as Tic and Dotcom; and 201B7, which is reported to have a poor propensity for hepatic differentiation [47]. We used a custom PCR array to assess gene expression in hepatoblast-like differentiated hPSCs that were induced using a slightly modified previously reported growth factor-mediated differentiation protocol [30,59]. The gene list of the array included markers associated with undifferentiated stem cells, endoderm, and hepatic differentiation. Differentiation of each cell line was scored according to the expression levels of genes associated with endoderm and hepatic differentiation lineage in the custom PCR array (Table 1). The gene expression profiles showed that H9, Dotcom, and Tic had higher scores than 201B7 or Squeaky (H9, Dotcom, Tic >> Squeaky, 201B7), as previously described [34,35,37].

Differentially expressed genes in hepatoblast-like cells derived from hPSCs

To compare characteristics of hPSC-derived hepatoblast-like cells, genes that were differentially expressed between hepatoblast-like differentiated and undifferentiated hPSC lines were examined using a custom PCR array analysis of undifferentiated hPSCs. The gene expression profiles of undifferentiated hPSCs, hPSC-derived hepatoblast-like differentiated cells, HepG2 cells, primary human fetal hepatocytes, and human adult hepatocytes were analyzed using two-way cluster analysis (Fig. 1). This analysis indicated that cells were divided into the following three groups: ''undifferentiated hPSCs'' (undifferentiated state of Dotcom, 201B7, Squeaky, H9, and Tic), ''differentiated hPSCs'' (hepatoblast-like cells from Dotcom, 201B7, Squeaky, H9, and Tic), and ''hepatocytes'' (HepG2, fetal hepatocytes, and adult hepatocytes). Differentially expressed genes were divided into four groups, designated clusters A, B, C, and D. Among them, the ''hepatocyte'' group expressed comparatively higher levels of clusters A and B genes. Further analysis of cluster B genes indicated that all ''differentiated hPSCs'' studied here could not be distinguished from ''hepatocytes'' (Supplementary Fig. S2). Therefore, expression of the 15 cluster B genes did not differ significantly among ''differentiated hPSCs.'' Next, expression of cluster A genes analyzed by two-way cluster analysis indicated that cells were clearly separated into two groups (Fig. 2). Two ''differentiated hPSCs,'' 201B7- and Squeaky-derived differentiated cells, belonged to a group with ''undifferentiated hPSCs,'' whereas the other ''differentiated hPSCs,'' H9-, Tic-, and Dotcom-derived differentiated cells belonged to another group with ''hepatocytes.'' These results indicated that expression of cluster A genes differed among ''differentiated hPSCs.'' Thus, 201B7- and Squeaky-derived hepatoblast-like cells were less differentiated compared with the others.

Table 1. Scores of Endoderm- and Hepatocyte-Related Gene Expression in the Hepatic Differentiation of Each Human Pluripotent Stem Cell Line

	Cell line				
Gene	*H9*	*Tic*	*Dotcom*	*Squeaky*	*201B7*
ABCB11	1.13	1.74	16.65	4.57	1.00
ABCC1	0.71	0.60	1.51	1.29	1.00
ABCC2	17.24	4.26	2.52	1.92	1.00
ABCC3	1.83	2.12	2.62	2.58	1.00
ABCC6	9.28	3.97	3.67	0.84	1.00
ABCD4	1.92	2.05	2.43	2.24	1.00
ADH1A	2.00	0.11	0.45	1.48	1.00
AFP	121.43	85.86	33.45	13.31	1.00
ALB	131.11	71.44	43.21	2.10	1.00
ALDH1A3	0.77	0.51	0.39	1.24	1.00
ALDH8A1	1.70	1.09	2.98	3.69	1.00
ALDOB	284.26	36.83	11.09	2.90	1.00
APOA1	17.56	14.27	7.24	2.75	1.00
APOF	28.64	6.14	8.78	0.25	1.00
ASGR1	22.51	13.05	4.41	1.55	1.00
C3	2.61	1.01	1.52	12.28	1.00
CDKN1A	1.21	2.15	2.03	1.28	1.00
CEACAM1	2.60	4.55	3.40	4.39	1.00
CEBPA	6.31	0.64	1.57	0.69	1.00
CEBPB	1.18	0.44	1.34	1.76	1.00
CPS1	1.36	3.57	1.37	1.39	1.00
CTNNB1	1.03	0.68	1.22	2.19	1.00
CYP1A1	0.84	0.10	1.92	1.84	1.00
CYP1A2	4.16	4.72	4.07	1.90	1.00
CYP26B1	13.86	0.21	1.24	6.28	1.00
CYP27A1	0.55	0.52	1.96	2.52	1.00
CYP2B6	5.69	0.04	4.79	2.70	1.00
CYP2C19	6.70	2.04	1.50	2.02	1.00
CYP2C8	0.75	2.98	1.96	2.82	1.00
CYP2C9	217.80	4.66	153.61	60.35	1.00
CYP2D6	2.26	1.42	1.92	3.56	1.00
CYP2E1	0.54	0.84	0.48	0.10	1.00
CYP2R1	3.34	3.94	2.89	1.67	1.00
CYP2S1	1.09	0.59	1.89	3.29	1.00
CYP3A4	12.45	36.24	1.86	0.49	1.00
CYP3A5	4.55	4.20	1.71	1.14	1.00
CYP3A7	1.61	2.86	2.06	1.38	1.00
CYP4B1	39.43	19.38	0.69	1.31	1.00
CYP7A1	0.47	0.37	0.56	0.15	1.00
CYP7B1	2.76	5.28	3.01	3.81	1.00
DPP4	3.47	3.96	5.36	3.81	1.00
G6PC	182.56	62.11	6.93	1.53	1.00
GATA4	2.13	0.70	2.17	2.25	1.00
GJA1	1.43	2.71	3.35	1.97	1.00
GJB1	24.25	3.46	5.64	1.19	1.00
HNF1A	7.14	2.90	7.49	5.38	1.00
ITGA6	1.74	1.68	2.28	1.94	1.00
KRT18	1.46	0.97	1.43	1.73	1.00
KRT8	1.29	0.53	1.08	1.86	1.00
LST1	7.88	11.16	3.35	5.72	1.00
MET	2.48	2.18	2.04	2.35	1.00
NR1/2	1.65	1.23	0.86	0.59	1.00
NR1/3	5.44	2.20	2.28	3.06	1.00
PCK1	78.72	21.07	10.52	0.35	1.00
POR	1.06	0.47	1.17	1.78	1.00
PROX1	1.65	3.30	4.01	2.81	1.00
SERPINA1	37.54	46.04	33.90	18.32	1.00
SLCO4A1	5.13	0.13	0.22	2.01	1.00
SULT2A1	19.46	3.41	3.21	0.26	1.00
TAT	0.36	0.10	0.64	0.35	1.00
TDO2	3.02	4.37	4.15	0.62	1.00
TF	2.45	0.62	0.25	0.10	1.00
TGFBR2	0.92	0.98	1.93	1.53	1.00
TTR	13.04	12.81	8.69	4.42	1.00
UGT1A1	625.78	721.75	402.54	91.51	1.00
SCORE	2.61	2.20	2.28	1.94	1.00

The hPSC lines were treated with a previously reported growth factor-mediated differentiation protocol toward hepatoblast-like [30,59] cells with minor modifications. Gene expression in the hPSCs was analyzed using the custom PCR array (122 genes) including markers associated with undifferentiated stem cells, endoderm, and hepatic differentiation (Supplementary Table S4). Differentiation levels were scored according to the expression levels of genes (65 genes) associated with endoderm and hepatic differentiation lineage in the custom PCR array. The mean of gene expression levels of five independent experiments were used for the analysis.

hPSCs, human pluripotent stem cells; PCR, polymerase chain reaction.

Gene ranking of undifferentiated and differentiated hPSCs

We hypothesized that the ranking of cluster A gene expression levels in hPSC-derived hepatocyte-like cells (H9, Tic, Dotcom >> 201B7, Squeaky) may be associated with the ranking of other genes expressed in undifferentiated hPSCs. To investigate gene expression markers of similar ranking in undifferentiated hPSCs average linkage hierarchical clustering was performed for 122 genes in the custom PCR array in undifferentiated hPSCs, together with 22 cluster A genes in the hPSC-derived hepatoblast-like cells (Supplementary Figs. S3 and S5). Furthermore, cluster analysis was also performed for hepatic differentiation-associated genes and cell signaling-associated genes (348 genes, Supplementary Figs. S4 and S6) using Human PI3K-AKT Signaling Pathway RT^2 Profiler PCR Array, Human TGFβ/BMP Signaling Pathway RT^2 Profiler PCR Array, Human Growth Factors RT^2 Profiler PCR Array, and Human WNT Signaling Pathway RT^2 Profiler PCR Array in undifferentiated hPSCs, together with cluster A genes (22 genes) in differentiated hPSCs. Expression levels of target genes were normalized to housekeeping genes *GAPDH*, *B2M*, and *HPRT* because other housekeeping genes, *RPL13A* and *HGDC* were unstable. We found the expression patterns of six genes in undifferentiated hPSCs were clustered with cluster A genes in the hPSC-derived hepatoblast-like cells (*CYP2E1*, *HNF1A*, *HNF1B*, *HNF4A*, *ONECUT-1*, and *PDX-1*) (Supplementary Fig. S3), and eight cell signaling-associated genes in the undifferentiated hPSCs were positively associated with cluster A genes in the hPSC-derived hepatoblast-like cells (*CSF2*, *FASLG*, *FGF-1*, *FGF-17*, *FGF-7*, *MSTN*, *RHOU*, and *TYMP*) (Supplementary Fig. S4). Next, to investigate possible inverse ranking of the cells according to gene expression levels, cluster analysis was performed separately (Supplementary Figs. S5

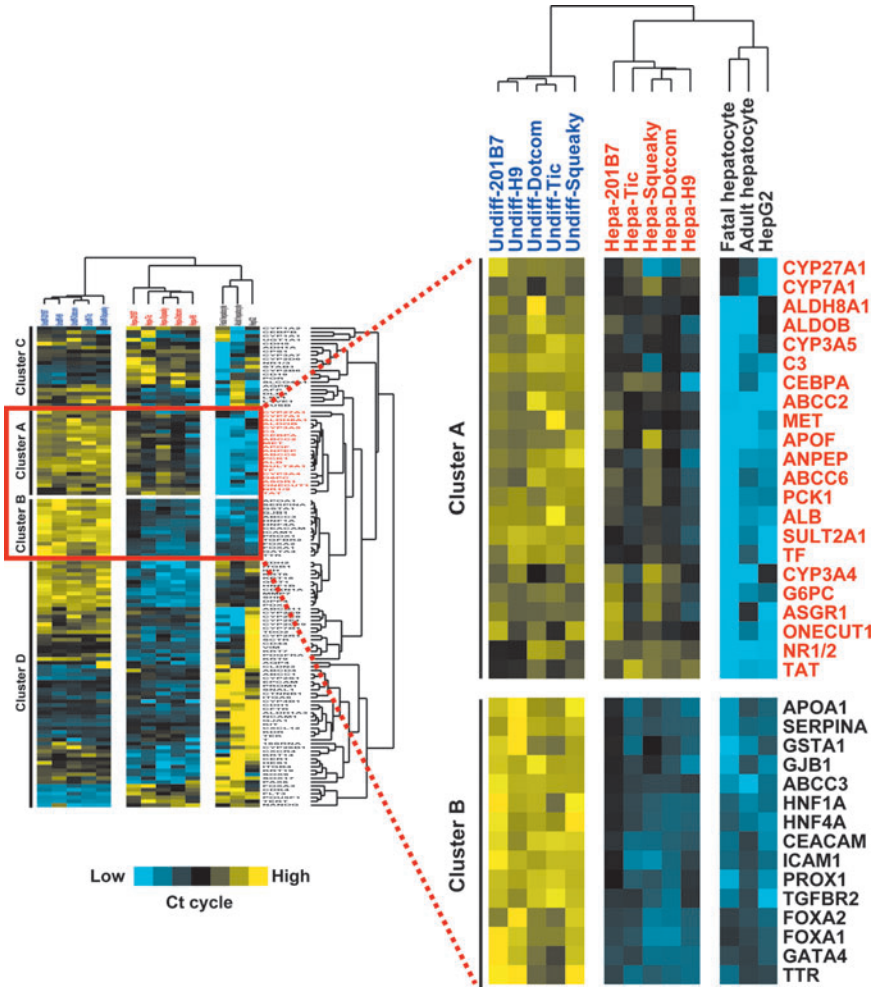

FIG. 1. Two-way cluster analysis of gene expression in undifferentiated and hepatocyte-like differentiated hPSC lines, HepG2, and primary fetal and adult hepatocytes. Thirteen cell types, undifferentiated (*blue*) and hepatocyte-like differentiated (*red*) hPSC lines, HepG2, and fetal (*black*) and adult human hepatocytes (*black*) were separated into three groups; 122 genes were separated into four groups, cluster A, cluster B, cluster C, and cluster D. *Yellow color* indicates high levels of gene expression, and *blue color* indicates low levels of gene expression. The mean of gene expression levels of five independent experiments were used for the analysis. hPSC, human pluripotent stem cell.

and S6). The expression of two genes in undifferentiated hPSCs was inversely correlated with cluster A genes in the hPSC-derived hepatoblast-like cells (*CXCL-12* and *TF*) (Supplementary Fig. S5) while the expression of four cell signaling-associated genes in undifferentiated hPSCs was inversely correlated with cluster A genes in the hPSC-derived hepatoblast-like cells (*IGF-1*, *PIK3R2*, *RHEB*, and *SFRP-4*) (Supplementary Fig. S6). Expression levels of these 20 genes were summarized in Table 2. The genes that were highly expressed (lower than 10 in ΔCt values) or differed significantly between hPSC lines (higher than 0.4 in SD values) were selected among the 20 genes. Then, three genes, *fibroblast growth factor-1* (*FGF-1*), *ras homolog family member U* (*RHOU*), and *thymidine phosphorylase* (*TYMP*), were chosen as candidate genes linking ranking of gene expression levels in the hPSC-derived hepatoblast-like cells.

Prediction of low hPSC differentiation propensity for hepatic lineage based on the expression of three candidate genes

To test whether these candidate genes could be used to predict low hepatic lineage differentiation propensity in hPSC lines, gene expression in 23 hPSC lines cultured under KSR-based or several serum-free culture conditions was measured by real-time PCR. ΔCt values of *FGF-1*, *RHOU*, and *TYMP* in the hPSC lines were arranged in ascending order in Supplementary Table S5. Among the 21 hPSC lines cultured under KSR-based culture conditions with MEF, ΔCt values of these three genes in hPSC lines were compared with those in H9, which is known to have a comparatively higher propensity [24,29–33,35–37,39,46] for hepatocyte differentiation, and 201B7, which is reported to have a low propensity [38,47]. Cell lines with ΔCt values higher than those of 201B7 were grouped into the lower gene expression group (red). Cell lines with ΔCt values lower than those of H9 were grouped into the higher gene expression group (blue). Next, cell lines grouped into the lower group were scored −1, whereas cells lines grouped into the higher group were scored +1. The total scores for the genes were calculated for each cell line, and the cell lines were arranged in ascending order in Table 3. Gene expression in 253G1B1 cells and Squeaky cells resembled that in 201B7 cells while the prediction score of the HES4 line, which is reported to have a high propensity for hepatic differentiation [47], was similar to that of H9. Interestingly, gene expression in the three MRC-5-derived lines, Squeaky, Dotcom, and Tic, closely resembled one another even though Dotcom and Tic exhibited higher hepatic differentiation propensities than Squeaky. Our gene expression data described above demonstrated that Tic differentiated into hepatoblast-like cells. Previous studies demonstrated that Dotcom and Tic differentiated well [34,35,37]. These

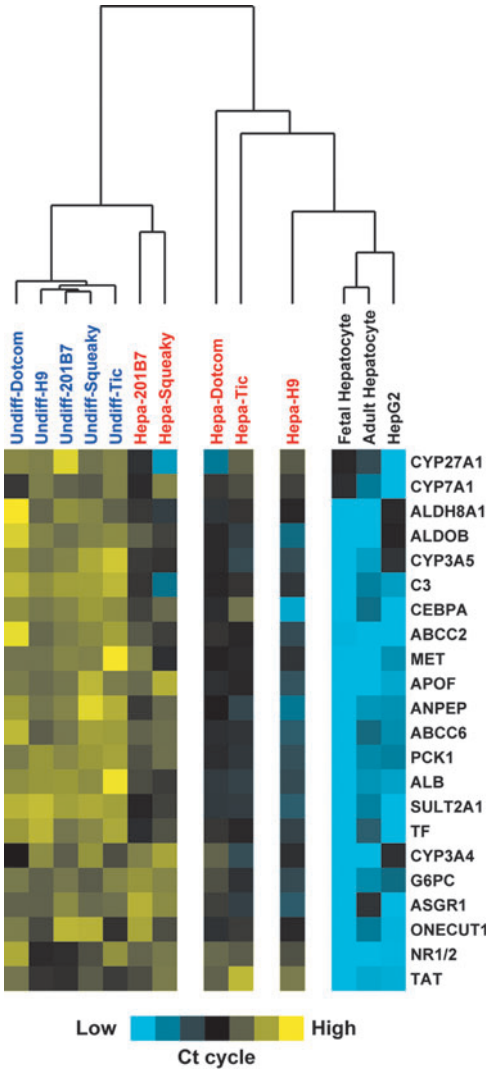

FIG. 2. Cluster analysis of 22 genes related to hepatic differentiation propensity in 13 cell types. The 22 genes were selected from 122 hepatocyte lineage-related genes, which distinguished gene expression in hepatoblast-like differentiated cells of five hPSCs. Hierarchical clustering of 13 cell types, undifferentiated hPSCs lines (*blue*), hPSC-derived hepatoblast-like differentiated hPSC lines (*red*), primary fetal (*black*) and adult human hepatocytes (*black*), and HepG2 (*black*) were separated into two groups. *Yellow color* indicates high levels of gene expression, and *blue color* indicates low levels of gene expression.

findings indicate that a prediction score lower than Tic's score is associated with low hepatic differentiation propensity, and they suggest that 253G1B1 cells would have a low propensity for hepatic lineage differentiation similar to 201B7 cells, which are known to have a low propensity for hepatocyte differentiation.

The ΔCt values of the three indicator genes in the hPSC lines H9, Tic, 253G1B1 and 201B7 cultured in xeno-free, feeder-free defined hESF-FX medium were compared with those in H9 or 201B7. The prediction score indicated that 253G1B1 may have a low propensity for hepatic differentiation. Comparison of 19-9-7T and its aberrant subclone (19-9-7Tv) cultured under feeder-free conditions using mTeSR1 medium and Matrigel showed that 19-9-7Tv may have lower propensity for hepatic differentiation compared with the parent line although the prediction score was not calculated with H9 or 201B7 cultured in the same conditions.

Propensity of hiPSC lines for hepatocyte differentiation

To further test the predictions for low hepatic differentiation, three high scoring cell lines (H9, KhES3, and KhES4) and two poorly scoring cell lines (253G1B1 and 201B7) were tested for hepatocyte differentiation. 19-9-7T hPSC and its aberrant subclone were also tested.

When maintained under the KSR-based conventional culture conditions with feeder cells, the prediction scores suggested that hepatic differentiation of KhES3 or KhES4 was higher than that of 201B7. H9, KhES3, KhES4, and 201B7 hPSC lines maintained under KSR-based culture conditions were tested for hepatocyte differentiation using the protocol described under Materials and Methods. Morphologies of the differentiated cells are shown in Fig. 3A. Hepatocyte differentiation was measured by albumin secretion, urea production, and CYP3A4 activity (Fig. 3B). By these criteria KhES3 and KhES4 gave rise to more differentiated hepatocyte-like cells than did 201B7 cells.

For cells cultured in hESF-FX the prediction scores suggested that the hepatic differentiation propensity of 253G1B1 was lower than that of H9. We differentiated H9, 253G1B1, and 201B7 cells cultured in hESF-FX medium into hepatocyte-like cells. The differentiated cells were immunostained with antibodies for the hepatocyte markers CYP3A4, albumin and AAT. 253G1B1-derived differentiated cells were partially immunostained with antibodies for CYP3A4, albumin, and AAT whereas 201B7-derived differentiated cells were very poorly stained by these antibodies (Supplementary Fig. S7). Albumin secretion of 253G1B1-derived cells was higher than that of 201B7-derived cells but lower than that of H9-derived cells (Fig. 3C). Differentiation levels were also determined by gene expression analysis using the custom PCR array (Fig. 3C and Supplementary Table S6): Hepatocyte markers, such as *ALB* and *G6PC*; *ATP-binding cassette transporters genes*, such as *ABCB11* and *ABCC2*; *Cytochrome P450 genes*, such as *CYP1A1*, *CYP1A2*, and *ASGR1*; *ATT* and *GSTA1* in 253G1-derived cells was higher than in 201B7-derived cells but lower than in H9-derived cells. Hepatic differentiation of 19-9-7T and its aberrant clone, which were established and maintained in mTeSR1 on Matrigel, was also tested. The prediction scores suggested that hepatic differentiation of the aberrant subclone was lower than that of parent line. In fact, albumin secretion, urea production, and CYP3A4 activity of the differentiated aberrant clone were lower than in the differentiated parent clone (Fig. 3D). Our data suggest that low prediction scores based on the expression of the *FGF-1, RHOU,* and *TYMP* genes correlate well with low propensity for hepatic differentiation.

Discussion

In this study we compared expression of genes associated with hepatic differentiation and cell signaling in five undifferentiated hPSCs and their hepatoblast-like

Table 2. The Value of ΔCt for Hepatocyte Lineage- and Signal- Related Gene Expression in Undifferentiated State of Each Human Pluripotent Stem Cell Lines

Order correlation 201B7 < H9							
	Cell line						
Gene	*201B7*	*Squeaky*	*Dotcom*	*Tic*	*H9*	*AV*	*SD*
Hepatocyte lineage-related gene							
CYP2E1	14.31	15.97	14.32	13.25	11.78	13.93	1.55
HNF1A	17.79	16.84	15.35	13.13	15.04	15.63	1.79
HNF1B	10.66	11.25	9.51	9.15	9.24	9.96	0.94
HNF4A	13.00	14.07	11.54	10.98	11.01	12.12	1.36
ONECUT1	10.03	9.98	9.13	7.66	8.15	8.99	1.07
PDX1	19.18	19.12	18.49	17.84	17.22	18.37	0.84
Signal-related gene							
CSF2	19.92	18.80	19.18	17.88	15.85	18.32	1.56
FASLG	19.87	19.61	19.04	19.05	17.68	19.05	0.85
*FGF-1**	9.23	8.85	9.26	8.68	7.45	8.69	0.74
FGF-17	11.50	11.61	10.63	9.84	9.56	10.63	0.93
FGF-7	18.24	18.26	18.32	18.17	17.88	18.17	0.17
MSTN	17.49	17.60	17.48	16.87	15.76	17.04	0.77
*RHOU**	5.67	5.74	4.86	4.62	4.79	5.14	0.52
*TYMP**	8.35	8.11	7.86	8.10	7.10	7.90	0.48
Inverse correlation 201B7 > H9							
	Cell line						
Gene	*201B7*	*Squeaky*	*Dotcom*	*Tic*	*H9*	*AV*	*SD*
Hepatocyte lineage-related gene							
CXCL12	2.11	2.25	2.20	2.44	2.82	2.36	0.28
TF	9.80	10.60	11.31	11.92	12.37	11.20	1.03
Signal-related gene							
IGF1	5.71	5.58	5.52	6.52	6.14	5.89	0.42
PIK3R2	3.97	3.72	3.80	4.12	4.03	3.93	0.16
RHEB	4.51	4.39	4.56	4.63	4.69	4.55	0.11
SFRP4	14.38	12.68	13.07	15.15	14.14	13.88	1.00

Genes that were positively and inversely correlated between undifferentiated hPSCs and hPSC-derived hepatoblast-like cells were summarized. Theses 20 genes were evaluated to lower than 10 in ΔCt values and higher than 0.4 in SD values. According to these criteria three genes, *FGF-1, RHOU,* and *TYMP* were selected as candidate genes (*). The mean of gene expression levels of five independent experiments were used for the analysis.

differentiated progeny to develop a method to predict low propensity of hPSC lines for hepatocyte differentiation. Bioinformatics analysis identified 20 genes that were differentially expressed between hPSC lines that differentiated well and poorly into hepatoblast-like cells. We selected three genes, *FGF-1, RHOU,* and *TYMP,* to test whether their low expression could be used to predict the low ability of hPSCs to differentiate along the hepatic lineage. We investigated expression of these three genes in 23 lines of undifferentiated hPSCs cultured under conventional KSR-based culture conditions or several feeder-free culture conditions, and we calculated prediction scores for hepatic differentiation. The validity of prediction scores of representative cell lines was confirmed by in vitro induction of hepatic differentiation. Our data suggest that low prediction scores were linked with low hepatic differentiation. A previous study reported the propensity for hepatic differentiation using similar hPSC cell lines and found that the propensity for hepatic differentiation decreased as follows: 201B6 > 201B2 > KhES3, H9 > KhES1 > 201B7, 253G1, when maintained under conventional culture conditions [47]. Our study found that differentiation prediction scores were H9, 201B2, 201B6, KhES3, Dotcom, and Tic >253G1B1 > 201B7. These results indicate that our analytical approach can reasonably predict the differentiation propensities of hPSCs.

It is widely understood that hPSC phenotypes are highly variable owing to differences in genomic DNA sequences, DNA methylation status, transcription efficiency, and cell signaling but also because of individual laboratories' culture conditions, culture methods, or culture techniques [41,65]. Accumulated differences may determine individual features of hPSC lines. In this study, we hypothesized that hepatocyte differentiation efficiency may differ according to variations in the gene expression profiles of undifferentiated hPSCs. Global gene expression analysis using microarray enables to evaluate stem cell phenotype with potential of lineage-specific differentiation [43,44]. Microarray analysis of brown adipocytes derived from wild-type and insulin receptor substrate knockout mice identified a gene expression pattern that predicts brown preadipocyte transition into adipocytes [66]. Because the microarray analyses generate a

Table 3. Comparison of Three Selected Gene Expression in Undifferentiated State of Each Human Pluripotent Stem Cell Line

Cutlure condition/Cell line	*TYMP*	*FGF-1*	*RHOU*	*H9 Point*	*201B7 point*	*Score*
KSR-based medium on feeders						
High scores						
H9	**8.5**	**7.05**	**7.22**	3	0	3
KhES-5	**7.85**	**6.84**	**6.98**	3	0	3
HES4	**8.13**	7.34	**5.41**	2	0	2
TIG120-4f1	**7.93**	8.49	**7.13**	2	0	2
KhES-2	**6.7**	7.15	7.63	1	0	1
KhES-4	**7.31**	8.28	7.31	1	0	1
201B2(subclone)	**6.37**	*9.8*	**6.78**	2	–1	1
Toe	9.56	9.01	**6.38**	1	0	1
UTA1	**8.37**	*9.67*	**5.85**	2	–1	1
KhES-1	**8.32**	9.09	7.84	1	0	1
201B6	8.95	8.79	7.76	0	0	0
KhES-3	8.92	8.84	7.54	0	0	0
201B2	**8.41**	*9.76*	7.53	1	–1	0
TIG114-4f1	**7.56**	*10.59*	7.43	1	–1	0
TIG108-4f3	**8.4**	*9.59*	7.64	1	–1	0
Dotcom	9.14	8.11	7.55	0	0	0
Tic	*10.55*	8.07	**7.16**	1	–1	0
Low scores						
Squeaky	*9.95*	7.16	8.36	0	–1	–1
253G1B1	9.69	*9.9*	*9.31*	0	–2	–2
201B7	*9.94*	*9.34*	*8.97*	0	–3	–3
hESF-FX medium without feeders						
High scores						
H9	**8.08**	**8.09**	**4.89**	3	0	3
Tic	8.4	*11.82*	**4.74**	1	–1	0
Low scores						
253G1B1	*9.81*	**7.86**	*6.62*	1	–2	–1
201B7	*9.33*	*10.01*	*6.25*	0	–3	–3
mTeSR1 medium without feeders						
19-9-7T normal	6.89	9.5	6.43	ND	ND	ND
19-9-7T abnormal	7.3	8.93	7.29	ND	ND	ND

The expression ratios listed in *bold* are lower than that of H9, and the ratios listed in *italic* are higher than that of 201B7.
The mean of gene expression levels of three independent experiments were used for the analysis.
KSR, knockout serum replacement; ND, not determined.

large amount of data, providing a tremendous number of candidate genes some of which are nonfunctional, a large of time, labor, and cost are required to identify objective genes. Therefore, we focused on genes previously reported to be associated with endoderm or hepatic differentiation pathways. Several hepatic differentiation protocols indicated that endoderm specification from hPSCs is driven by a combination of several growth factors including BMP, NODAL/ACTIVIN A, FGF, and WNT [13,26–31]. These growth factors stimulate intracellular signaling, such as MAPK, PI3K-AKT, ERK, and GSK3B signals. Therefore, we used a PCR array system including genes related to hepatocyte differentiation markers, PI3K-AKTsignaling pathway, TGFβ/BMP signaling pathway, growth factors, or WNT signaling pathway. From these gene expression profiles, three genes, *TYMP*, *FGF-1*, and *RHOU*, were selected as candidates that may predict the hepatic differentiation efficiency of hPSC lines. TYMP is also known as platelet-derived endothelial cell growth factor and plays a role in maintaining the integrity of blood vessels, promoting endothelial growth and angiogenesis in vivo, and promoting the chemotactic activity of endothelial cells in vitro [67]. TYMP expression is reported to be elevated in liver, lung, and breast tumors [68]. FGF-1, a member of the FGF family, is known to be involved in vertebrate embryonic and fetal development [69]. RHOU is implicated in the connection between β-catenin-dependent WNT signaling and cellular processes normally associated with WNT/PCP pathways [70]. These findings implicate these genes in the regulation of differentiation toward hepatocytes. We aim to further investigate the function of these genes in future studies.

Several highly efficient methods for inducing differentiation of hPSCs into hepatocytes have been proposed [39,71–74]. On the other hand, an enormous number of hiPSCs with variable phenotypes are generated from various donors in several institutes in the world. Ideally, hPSCs possess the potential to differentiate into cells of all three germ layers. However, in practice, hPSC differentiation propensity is limited to specific lineages. Developing a method to select good clones would be immensely useful. Several previous studies have proposed bioinformatic assay platforms capable of predicting hPSCs phenotypes [43,44]. These assays used embryoid bodies (EBs) to evaluate propensity to differentiate into the three germ layers. We first tried to

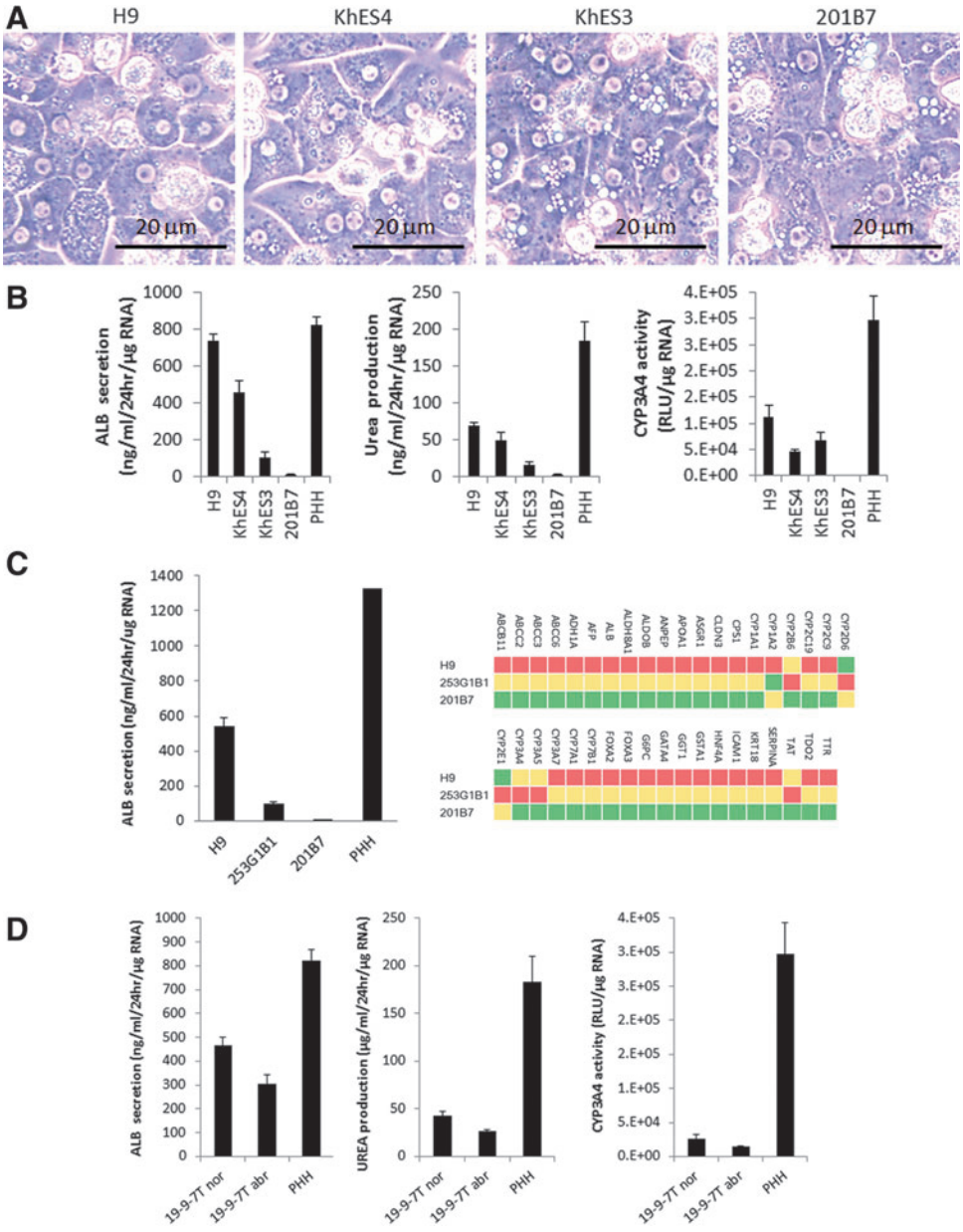

FIG. 3. Hepatic differentiation of the hPSC line predicted a low differentiation tendency. **(A)** Phase contrast images of hepatocyte-like cells derived from hPSCs, H9, KhES-4, KhES-4, and 201B7 maintained under conventional KSR-based culture condition with feeder cells were shown. Scale bars = 20 μm. **(B)** albumin secretion, urea production, and CYP3A4 activity of these cells and primary human hepatocytes (PHH) were examined. The results are presented as the mean of three biological independent experiments, and error bars represent SD. **(C)** *Left:* albumin secretion from hepatocyte-like cells derived from hPSCs H9, 253G1B1, and 201B7 cultured under feeder-free culture conditions were examined by ELISA on differentiation day 20. Concentration was calculated using a standard reagent followed by normalization to the total RNA content. The results are presented as the mean of three biological independent experiments, and error bars represent SD. *Right:* custom polymerase chain reaction array analysis of these hepatocyte-like cells. Each gene expression level is compared among three lines (*red* > *yellow* > *green*). The mean of gene expression levels of three independent experiments were used for the analysis. **(D)** Albumin secretion, urea production, and CYP3A4 activity of 19-9-7T, its aberrant clone (19-9-7T abr) and primary human hepatocytes (PHH) were examined. The results are presented as the mean of three biological independent experiments, and error bars represent SD. KSR, knockout serum replacement.

examine the propensity of five hPSC cell lines, H9, 201B7, Tic, Dotcom, and Squeaky, to differentiate into three germ layers using a Human Stem Cell Pluripotency Array, as described previously [41] (Supplementary Table S6). Expression of endoderm marker in EBs derived from Tic and Dotcom was higher than in Squeaky, and expression in H9 was similar to that in 201B7 (Supplementary Table S7). Stem cell marker expression was lower in H9, Tic, and Squeaky than in 201B7. However, scoring of genes associated with hepatic differentiation, *tyrosine aminotransferase* (*TAT*), *alpha-fetoprotein* (*AFP*), *forkhead box protein A2* (*FOXA2*), *serpin peptidase inhibitor, clade A member 1*

(*SERPINA1*; known as *AAT*), and *SRY-related HMG-box 17* (*SOX17*), indicated that autonomous hepatic differentiation propensity decreased from Tic > Dotcom >201B7 > H9 > Squeaky (Supplementary Table S8). However, 201B7 was previously reported to have a low hepatic propensity [47], whereas H9 is reported to have high hepatic propensity [27,30,31,75]. Our results were consistent with these reports [35,38], indicating that autonomous differentiation, by forming EB, is not associated with hepatocyte differentiation induced by the specific protocols we employed. Neural or mesoderm differentiation is autonomously promoted. On the other hand, endoderm differentiation requires high doses of specific growth factors at specific timing. It may be difficult to predict endoderm differentiation propensity based on EB gene expression data alone.

Conclusions

We hypothesized that hepatocyte differentiation efficiency of hPSC can be predicted by analyzing the gene expression profile in undifferentiated hPSCs. We were concerned that the large quantity of data generated by global gene microarrays may fail to highlight minute differences in hPSC phenotypes. Instead, we assessed the expression levels of selected genes previously reported to be associated with hepatic differentiation and cell signaling. Clustering analyses of five hPSC lines identified three candidate genes. In this study, we estimated the prediction scores for differentiation propensity of 23 hPSC clones compared with those of H9 or 201B7 hPSC lines. Our analytical approach proposed a method for prediction method using gene expression ranking and bioinformatic analysis. The reliability of this method could be assessed by examining more hPSC lines, and additional reliable markers could be identified. If hPSC gene expression profiles were provided by international public stem cell banks or related platforms, it may be possible to identify more hPSC lines with lower hepatic differentiation propensity.

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Author Disclosure Statement

No competing financial interests exist.

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