Differentiation of human induced pluripotent stem cells in William's E initiation medium supplemented with 3-bromopyruvate and 2-deoxy-D-glucose

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Abstract. Hepatocyte selection medium (HSM) is deprived of glucose and supplemented with galactose, and is based on Leibovitz's-15 (L15) medium. HSM may promote the differentiation of human induced pluripotent stem (iPS) cells towards hepatocyte lineage. These culture conditions result in increased expression of galactokinase (GALK)-1 and GALK2. However, iPS cells do not survive in HSM. Two potential alternatives to glucose deprivation are treatment with 3-bromopyruvate (3BP), an analogue of pyruvate, and 2-deoxy-D-glucose (2DG), an analogue of glucose. The promoters of *GALK1* and *GALK2* were subcloned using the pMetLuc2 reporter plasmid to make pMetLuc2/*GALK1* and pMetLuc2/*GALK2*, respectively. 201B7 human iPS cells were transfected with the reporter plasmids, cultured in HSM and analyzed by luciferase assay. Furthermore, 201B7 cells were cultured in L15, William's E (WE) or Dulbecco's modified Eagle's medium/nutrient mixture F-12 Ham (DF12) supplemented with 3BP, 2DG or a combination of the two, for 15 days, and subjected to reverse transcription-quantitative polymerase chain reaction to measure the levels of α-fetoprotein (*AFP*) mRNA expression. *Metridia* luciferase activity was significantly higher in cells cultured in HSM compared with those in ReproFF medium ($P<0.05$). 3BP and 2DG treatment, alone or in combination, decreased *AFP* expression levels in cells cultured in L15 and DF12. The combination of 3BP+2DG increased the expression levels of *AFP* in WE. Without 3BP or 2DG, *AFP* expression was higher in L15 compared with WE or DF12. The promoters of *GALK1* and *GALK2* were activated in 201B7 cells cultured in HSM, enabling survival using galactose as an energy source. 3BP and 2DG supplementation in WE medium may promote the differentiation of iPS cells to the hepatocyte lineage.

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Introduction

Human induced pluripotent stem (iPS) cells are a promising cell source for differentiation to somatic cells that are transplantable to recipients (1,2). Liver failure is a fatal disease that is characterized by a decrease in functioning hepatocytes (3). Transplanted hepatocytes differentiated from iPS cells may be a potential cure for patients with liver failure. Currently, hepatocytes are differentiated by growth factor stimulation or the introduction of transcription factors (4-9); using current protocols, hepatocytes remain in an immature state (10).

Glucose is a crucial source of energy for cells. Unlike iPS cells, hepatocytes can survive in hepatocyte selection medium (HSM) lacking glucose but supplemented with galactose (11). Galactokinases (GALKs) metabolize galactose to galactose-1-phosphate, which enters glycolysis (12). In humans, there are two forms: GALK1 and GALK2 (13). The expression levels of GALK1 and GALK2 increase in iPS cells grown in a modified HSM: hepatocyte differentiation inducer (HDI) (14). These data suggest that galactose may be used as an energy source instead of glucose by iPS cells cultured in HSM. Unexpectedly, the expression levels of α-fetoprotein (AFP), a marker of immature hepatocytes, were increased in iPS cells cultured in HSM and HDI (11,14). These data suggest that hepatocyte differentiation may be initiated in glucose-free media that is supplemented with galactose.

One major problem is that iPS cells do not survive in HSM and HDI beyond 3 and 7 days, respectively (14). 2-Deoxy-D-glucose (2DG) is an analogue of glucose that is taken up by cells but is not metabolized (11,15). 3-Bromopyruvate (3BP) is an analogue of pyruvate, which is the final product of glycolysis and which enters the citric acid cycle (16,17). 2DG may be used as a model of glucose deprivation. The present study therefore investigated the effects of 2DG and 3BP on iPS cells with respect to hepatocyte differentiation.

Table I. Primer sequences.

Gene (GenBank accession no.)	Primer name	Sequence (5'→3')	Product size (bp)
AFP (NM_001134)	OMC317	F: ACACAAAAAGCCCACTCCAG	147
	OMC318	R: GGTGCATACAGGAAGGGATG	147
RPL19 (BC095445)	OMC321	F: CGAATGCCAGAGAAGGTCAC	157
	OMC322	R: CCATGAGAATCCGCTTGTTT	157

AFP, α-fetoprotein; bp, base pairs; F, forward; R, reverse; RPL19, ribosomal protein L19.

Materials and methods

Cell culture. The human iPS cell line 201B7 was purchased from the RIKEN Cell Bank (Tsukuba, Japan) and cultured under feeder-free conditions in ReproFF medium (ReproCELL, Inc., Yokohama, Japan) on 10-cm dishes (Asahi Glass Co. Ltd., Tokyo, Japan) coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). The cells were incubated at 37˚C in a humidified chamber with 5% CO_2. They were then harvested with Accutase (Innovative Cell Technologies, Inc., San Diego, CA, USA), and spread onto individual 10-cm dishes at a density of $1x10^6$ cells/dish, and passaged every 4-5 days.

Culture in conventional media with or without 3BP or 2DG. 201B7 cells were harvested and spread onto 6-well plates (Asahi Glass Co. Ltd.) at a density of $1x10^6$ cells/well and cultured in 5% of carbon dioxide at 37˚C in ReproFF, Leibovitz's-15 (L15), William's E (WE) or Dulbecco's modified Eagle's medium/nutrient mixture F-12 Ham (DF12). Repro FF was purchased from ReproCELL Inc., all other media were purchased from Life Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA). ReproFF, WE, and DF12 all contained glucose, whereas L15 contained. galactose. L15, WE and DF12 media were supplemented with nicotinamide (1.2 mg/ml), proline (30 ng/ml) and 10% KnockOut Serum Replacement (Life Technologies; Thermo Fisher Scientific, Inc.) medium supplement. Nicotinamide (1.2 g/l) and proline (260 mM) were added as they are necessary for primary hepatocyte proliferation (18,19). For certain experiments, 3BP (10 μM; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) or 2DG (10 μM; Sigma-Aldrich; Merck KGaA) was added. Following 7 days culture, the cells were observed by light microscopy (CKX41N-31PHP; Olympus Corporation, Tokyo, Japan) without any treatment or subjected to reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

HSM. HSM was prepared from amino acid powders using the formulation of L15 medium (Life Technologies; Thermo Fisher Scientific, Inc.), but omitting arginine, tyrosine, glucose and sodium pyruvate, and with the addition of galactose (900 mg/l), ornithine (1 mM), glycerol (5 mM) and proline (260 mM; all from Wako Pure Chemical Industries, Ltd., Osaka, Japan) (11). Knockout Serum Replacement (Life Technologies; Thermo Fisher Scientific, Inc.) was used in place of fetal bovine serum to establish defined xeno-free conditions and was added at a final concentration of 10%.

RT-qPCR. Total RNA (5 μg) was isolated from 201B7 cells using Isogen (Nippon Gene Co., Ltd., Tokyo, Japan) and subsequently used for the synthesis of first-strand cDNA with SuperScript III Reverse Transcriptase and oligo (dT) primers (Life Technologies; Thermo Fisher Scientific, Inc.), following the manufacturer's protocols. Total RNA from the human fetal liver was purchased from Clontech Laboratories, Inc. (Mountain View, CA, USA) to serve as a positive control. qPCR was performed using Fast SYBR-Green Master Mix (Life Technologies; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol and the results were analyzed using the MiniOpticon Real-Time PCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). qPCR was performed for 40 cycles of two steps, consisting of 5 sec denaturation (95˚C) and 5 sec annealing-extension (60˚C). Primer sequences are presented in Table I. The constitutively expressed housekeeping gene ribosomal protein L19 (*RPL19*) was used as an endogenous internal control to monitor the levels of mRNA expression (20). mRNA expression levels were analyzed automatically using the MiniOpticon system based on $2^{-\Delta\Delta Cq}$ method (21). The relative expression was calculated as the expression level of a specific gene divided by that of *RPL19*. Experiments were repeated three times.

Plasmid construction. The *Sac*I-*Hin*dIII fragments of the *GALK1* and *GALK2* promoters, contained within pLightSwitch Promoter vectors (SwitchGear Genomics, Carlsbad, CA, USA) were subcloned into the pMetLuc2-reporter vector (Promega Corporation, Madison, WI, USA) to make pMetLuc2/*GALK1* and pMetLuc2/*GALK2* reporter plasmids, respectively. GALK1 or GALk2 promoters (2 μg) in pLightSwitch Promoter or pMetLuc2-reporter vector, was digested with 10 U of *Sac*I in a volume of 10 μl at 37˚C for 1 h. The digested samples were mixed with 10 U of *Hin*dIII, the total volume was 50 μl. The digested fragments were fractionated with gel electrophoresis, and purified with a gel extraction kit (Qiagen GmbH, Hilden, Germany). The purified fragments were subcloned into the digested pMetLuc2-reporter with a ligation kit (Takara Bio. Inc., Otsu, Japan). The inserted fragments were confirmed by sequencing (Riken Genesis, Co. Ltd., Tokyo, Japan).

Transfection and Metridia *luciferase activity assay*. 201B7 cells were plated on 96-well plates (Asahi Glass Co. Ltd.) coated with Matrigel, at a density of $5x10^5$ cells/well. pMetLuc2-control, pMetLuc2/*GALK1* and pMetLuc2/*GALK2* were transfected with FuGENE HD Transfection Reagent

(100 ng/well; Clontech Laboratories, Inc.) according to the manufacturer's protocol (22). The reporter plasmids expressed *Metridia* luciferase, which is secreted into the medium; the pMetLuc2-control plasmid expresses *Metridia* luciferase driven by the cytomegalovirus (*CMV*) immediate early promoter. A luciferase assay was performed following 2 days of culture in ReproFF, WE or HSM using a Ready-To-Glow Secreted Luciferase Reporter assay (Clontech Laboratories, Inc.) and a Gene Light GL-200A luminometer (Microtec Co. Ltd., Funabashi, Japan). To monitor transfection efficiency, 10 ng of the pSEAP2 control vector (Clontech Laboratories, Inc.) was added to each well of the black 96-well plates and transcriptional activity was measured using a Secreted Embryonic Alkaline Phosphatase (SEAP) Chemiluminescence kit (Clontech Laboratories, Inc.) and the Gene Light luminometer according to the manufacturer's protocols. Luciferase activity was calculated as the *Metridia* luciferase activity divided by the SEAP activity (relative light units). The *Metridia* luciferase activity was normalized against that of the *CMV* promoter. The *Metridia* luciferase activity is presented in relative light units. The experiments were repeated three times.

Statistical analysis. Relative expression levels of *AFP* and relative light units of *Metridia* luciferase were analyzed by a one-way analysis of variance using JMP version 5.0J (SAS Institute, Inc., Cary, NC, USA), followed by Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

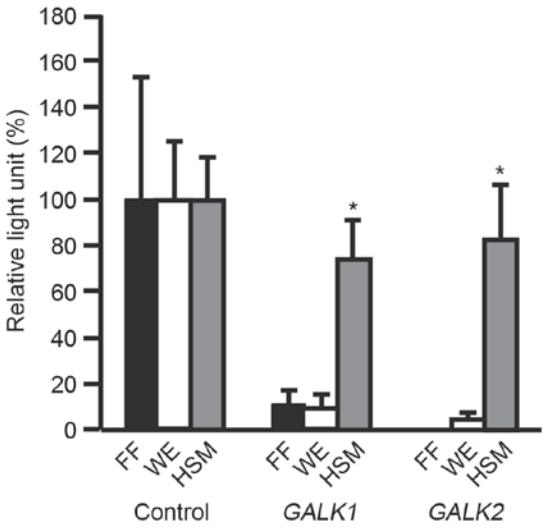

Figure 1. *Metridia* luciferase assay. 201B7 human induced pluripotent stem cells were transfected with a reporter plasmid expressing *Metridia* luciferase driven by the (*CMV*) immediate early promoter, the *GALK1* promoter or the *GALK2* promoter, and cultured in FF, WE medium or HSM. The pSEAP2 control vector secretes embryonic alkaline phosphatase and was co-transfected to monitor the transfection efficiency with 1/10 of the amount of the above reporter plasmids. The relative *Metridia* luciferase activity was calculated as the *Metridia* luciferase activity divided by that of SEAP. The cells were subjected to a luciferase assay following 2 days of culture. The *Metridia* luciferase activity was normalized against that of the *CMV* promoter. Results are presented as the mean ± standard deviation; *P<0.05 vs. FF in each condition; n=4. CMV, cytomegalovirus; FF, ReproFF medium; *GALK*, galactokinase; HSM, hepatocyte selection medium; SEAP, secreted embryonic alkaline phosphatase; WE William's E medium.

Results

To examine changes in the promoter activities of *GALK1* and *GALK2* in medium that lacks glucose and is supplemented with galactose, HSM was used to culture 201B7 iPS cells. The 201B7 cells were transfected with pMetLuc2-control, pMetLuc2/*GALK1* or pMetLuc2/*GALK2* reporter plasmids, and cultured in ReproFF, WE or HSM medium. The cells were subjected to a *Metridia* luciferase assay (Fig. 1). Metridia luciferase activity of GALK1 or GALK2 was normalized against that of CMV. WE medium was used as it was originally established for primary hepatocyte culture (23,24). Metiridia luciferase activity was significantly higher in HSM compared with WE (P<0.05). *Metridia* luciferase activity was significantly higher in HSM compared with ReproFF (P<0.05).

The effects of 3BP and 2DG on the morphological features of 201B7 cells were analyzed by culturing cells in L15, WE or DF12, with or without 3BP or 2DG (Fig. 2). The morphology of cells grown in HSM was not assessed as these cells died within three days. Following 7 days of culture, the cells were observed under a microscope and imaged. No significant morphological differences were observed when compared with the morphology of cells cultured in ReproFF.

The differentiation of 201B7 cells to the hepatocyte lineage was examined in cells that were cultured in L15, WE or DF12 with or without 3BP or 2DG. AFP expression in HSM-cultured cells was not analyzed, because these cells died within three days. Following 7 days of culture, the cells were subjected to RT-PCR to analyze *AFP* mRNA expression (Fig. 3). The expression levels of *AFP* were dependent on the culture medium as well as on the effects of 3BD and 2DG treatment. Treatment with 3BP, 2DG or 3BP+2DG decreased the expression levels of *AFP* in 201B7 cells cultured in L15 or DF12 media, compared with untreated cells. The combination of 3BP+2DG treatment resulted in an increase in *AFP* expression levels in WE medium. *AFP* expression was higher in cells cultured in unsupplemented L15 medium compared with untreated WE and DF12 media.

Discussion

A previous study demonstrated that the expression levels of GALK1 and GALK2 are increased in iPS cells cultured in HDI that lacks glucose and is supplemented with galactose (14). These data suggest that glucose deprivation and galactose supplementation affect the transcription of *GALK1* and *GALK2*. However, to the best of our knowledge, the promoter activities of *GALK1* and *GALK2* have not been previously analyzed. In the present study, a *Metridia* luciferase assay demonstrated that the *GALK1* and *GALK2* promoters were activated in 201B7 iPS cells cultured in HSM. Unlike HDI, HSM does not have added growth factors or small molecules. HSM was suitable for the investigation of the *GALK1* and *GALK2* promoter activities in medium without glucose and supplemented with galactose. The data from the present study clearly demonstrated that the promoters of *GALK1* and *GALK2* were activated in 201B7 cells cultured in a medium without glucose and supplemented with galactose.

Glucose deprivation and galactose supplementation were expected to promote the differentiation of iPS cells to the

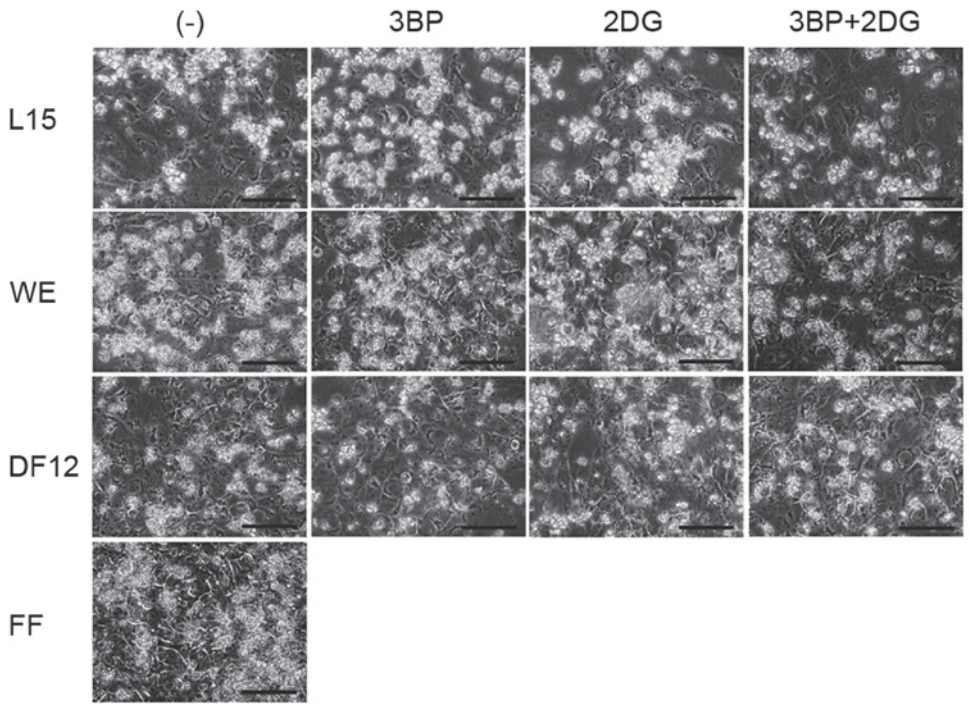

Figure 2. Morphological analysis of 201B7 human induced pluripotent stem cells in various culture conditions. 201B7 cells were initially cultured in FF, then the medium was changed to L15, WE or DF12 medium with or without 3BP (10 μM), 2DG (10 μM) or a combination of the two (3BP+2DG). Images were captured following 7 days of culture. Original magnification, x400; scale bar, 100 μm. 2DG, 2-deoxy-D-glucose; 3BP, 3-bromopyruvate; DF12, Dulbecco's modified Eagle's medium/nutrient mixture F-12 Ham; FF, ReproFF; L15, Leibovitz's-15; WE, William's E.

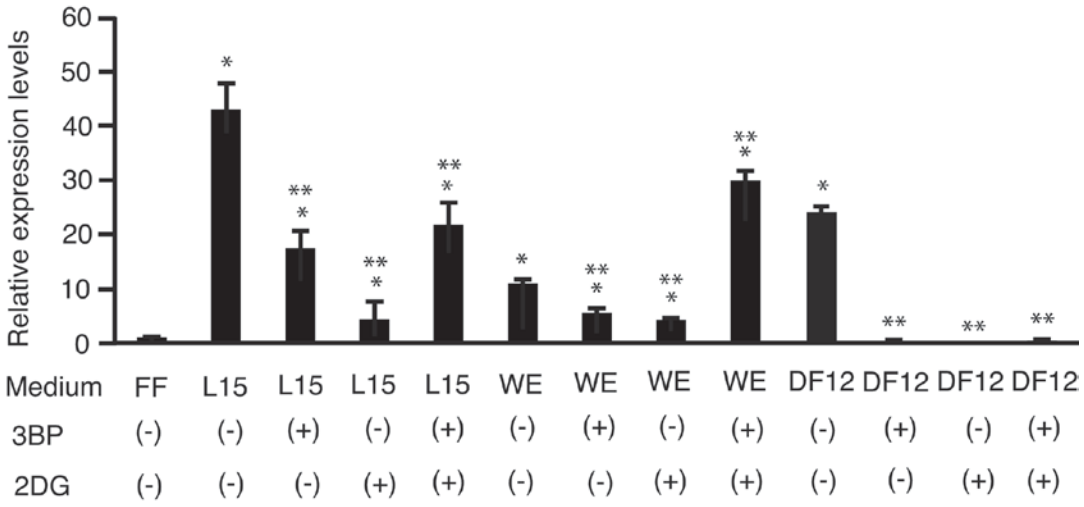

Figure 3. Reverse-transcription-quantitative polymerase chain reaction. 201B7 human induced pluripotent stem cells were cultured in L15, WE or DF12 with (+) or without (-) 3BP (10 μM) or 2DG (10 μM). RNA was isolated and subjected to reverse transcription-quantitative polymerase chain reaction to analyze the expression levels of α-fetoprotein. 201B7 cells cultured in FF were used as a control. Results are presented as the mean ± standard deviation; *P<0.05 vs. FF; **P<0.05, vs. 3BP (-) and 2DG (-); n=3. 2DG, 2-deoxy-D-glucose; 3BP, 3-bromopyruvate; DF12, Dulbecco's modified Eagle's medium/nutrient mixture F-12 Ham; FF, ReproFF; L15, Leibovitz's-15; WE, William's E.

hepatocyte lineage. One major problem is that iPS cells do not survive in HSM and HDI beyond 3 and 7 days, respectively (14). To overcome this limitation, the present study used 3BP and/or 2DG treatment to investigate an alternative to glucose deprivation and galactose supplementation. The combination of 3BP+2DG increased *AFP* mRNA expression levels in 201B7 cells cultured in WE medium, but not in DF12. It was not clear why the changes in *AFP* expression levels differed in magnitude between the three 3BP and/or 2DG treatments. Furthermore, it was not clear why AFP was not detected in any of the DF12 3BP/2DG treatment groups. Cells cultured in L15 medium without 3BP or 2DG treatment exhibited increased expression levels of *AFP*. L15 does not contain glucose, but includes galactose. Accordingly, these results suggested that glucose deprivation and supplementation with galactose promoted the differentiation of iPS cells to the hepatocyte lineage. WE medium was originally established for primary hepatocyte culture (25). The highest expression was observed

in untreated L15, however, iPS cells decrease in number after seven days of culture in L15 (26). WE medium supplemented with 3BP+2DG may be suitable for the differentiation of iPS cells to the hepatocyte lineage.

One major limitation of the present study was that the differentiation of iPS cells to a hepatocyte lineage was only confirmed via the measurement of AFP mRNA expression levels. Future experiments should verify hepatocyte differentiation using functional tests, such as indocyanine green up-take. In conclusion, the promoters of *GALK1* and *GALK2* were activated in 201B7 cells cultured in HSM. However, a major problem with HSM is that cultured iPS cells die within three days; 3BP and 2DG in WE may therefore be suitable for differentiation of iPS cells to hepatocyte lineages instead of HSM, and future studies should further investigate cell viability and differentiation into a hepatocyte lineage, following treatment with these compounds.

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