

2-Deoxy-D-glucose initiates hepatocyte differentiation in human induced pluripotent stem cells

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Abstract. To initiate hepatocyte differentiation in human induced pluripotent stem (iPS) cells, cells are cultured in a medium lacking glucose but supplemented with galactose (hepatocyte selection medium, HSM) or in medium supplemented with oncostatin M and small molecules (hepatocyte differentiation inducer, HDI). In the present study, 2-Deoxy-D-glucose (2DG), an analogue of glucose, was utilized instead of glucose deprivation and the effect of 2DG supplementation on iPS differentiation was examined. First, 201B7 cells, an iPS cell line, were cultured in HSM or HDI media for 2 days and then subjected to reverse transcription-quantitative polymerase chain reaction (RT-qPCR) in order to analyze expression levels of established hepatocyte markers, including cytosolic aspartate aminotransferase (AST), mitochondrial AST, alanine aminotransferase (ALT), and glyceral kinase. mRNA expression levels of mitochondrial AST, ALT, and glycogen synthase significantly increased following culture in HSM and HDI compared with ReproFF media. Cytosolic AST mRNA expression levels significantly increased following culture in HDI compared with ReproFF media, but not in HSM. To test the effect of 2DG on iPS differentiation, 201B7 cells were cultured in ReproFF, a feeder-free medium that retains pluripotency, supplemented with 2DG. Following 7 days of culture, the cells were subjected to RT-qPCR to analyze expression levels of α -fetoprotein (AFP), a marker of immature hepatocytes. AFP mRNA expression levels significantly increased with the addition of 0.1 μ M 2DG in the media, and galactose addition acted synergistically with 2DG to further upregulate AFP expression. In conclusion, the present study demonstrated that hepatocyte differentiation was initiated in iPS cells cultured in HSM and HDI media and

that 2DG could be used as a supplement instead of glucose deprivation to initiate hepatocyte differentiation in iPS cells.

Introduction

Hepatic failure, a condition where the number of functioning hepatocytes significantly decreases, is fatal (1). Transplantation of hepatocytes in patients with liver failure could be a promising treatment. Human induced pluripotent stem (iPS) cells are established with the introduction of reprogramming factors (2,3). iPS cells are an ideal source for the generation of hepatocytes. The current protocols for generating hepatocytes from iPS cells consist of sequential stimulation with growth factors and introduction of transcription factors (4-9). The generated hepatocytes remain in an immature state, namely hepatic progenitor-like cells (10).

Aspartate aminotransferase (AST) is expressed in the cytosol (cytosolic AST) and mitochondria (mitochondrial AST) in myocytes and hepatocytes (11). AST catalyzes the interconversion of aspartate and α -ketoglutarate to glutamate and oxaloacetate. Alanine aminotransferase (ALT) is mainly expressed in hepatocytes and catalyzes the conversion of pyruvate and glutamate to L-alanine and α -ketoglutarate (12). Glycogen synthase synthesizes glycogen from glucose metabolites (13). If a cell expresses AST, ALT, and glycogen synthase, it is considered to have a metabolic function specific to that of hepatocytes.

Glucose is indispensable for the survival of cells. Hepatocytes express galactokinase, an enzyme that catalyzes the conversion of galactose to galactose-1-phosphate. Galactose-1-phosphate enters the glycolytic pathway and is used as a source of energy by the cells. Hepatocyte selection medium (HSM), a medium that lacks glucose but contains galactose, is used to eliminate iPS cells and specifically select for hepatocytes (14). Hepatocytes survive in HSM because they express galactokinase, allowing them to utilize the galactose in the medium for their energy needs. Expression of α -fetoprotein (AFP), a marker of immature hepatocytes, is increased in iPS cells cultured in HSM (15), suggesting that glucose deprivation and galactose supplementation promote hepatocyte differentiation of iPS cells.

2-Deoxy-D-glucose (2DG) is an analogue of glucose (16), which is taken up by cells similar to glucose. However, unlike

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glucose, it is not metabolized and it is accumulated in the cells. 2DG is used in positron emission tomography for diagnostic imaging of cancer (17). It is hypothesized that if 2DG was added in a medium, the cultured cells would take up 2DG similar to glucose, but the cells would not be able to metabolize 2DG as a source of energy. Such addition of 2DG in the medium would therefore be similar to glucose deprivation. In the present study, the effect of galactose and 2DG was examined on hepatocyte differentiation of iPS cells, by evaluating the expression of AFP, as a marker of immature hepatocytes. In addition, the expression levels of AST, ALT, and glycogen synthase in iPS cells cultured in HSM or hepatocyte differentiation inducer (HDI) were examined.

Materials and methods

Cell culture. A human iPS cell line, 201B7 (Riken BioResource Center, Tsukuba, Japan), was cultured on 10 cm dishes coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) in ReproFF medium (ReproCELL, Inc., Yokohama, Japan) at 37°C with 5% carbon dioxide in a humidified chamber. Once confluent, the cells were rinsed with PBS and harvested using Accutase (Innovative Cell Technologies, Inc., San Diego, CA, USA). The cells were then observed under a microscope (CKX41N-31 PHP; Olympus Corporation, Tokyo, Japan).

Reagents. Non-essential amino acids solution (NEAA; 100x) and sodium pyruvate (100x) were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). The apoptosis inhibitor M50054 [2,2'-methylenebis (1,3-cyclohexanedione)] was purchased from Merck KGaA (Darmstadt, Germany). FPH1 [2-(N-(5-chloro-2-methylphenyl) methylsulfonamido)-N-(2,6-difluorophenyl) acetamide] was purchased from Xcessbio Biosciences, Inc. (San Diego, CA, USA) (18). Galactose, ornithine, glycerol, oncostatin M, nicotinamide, proline and L-glutamine were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 2DG was purchased from Sigma-Aldrich; Merck KGaA.

HSM and HDI media. 201B7 cells were cultured in 6-well plates coated with Matrigel (BD Biosciences) in HSM or HDI for 2 days and then subjected to reverse transcription-quantitative polymerase chain reaction (RT-qPCR). HSM consisted of Leibovitz's-15 (L15) medium (Thermo Fisher Scientific, Inc.), with the omission of arginine, tyrosine, glucose, and sodium pyruvate and the addition of galactose (900 mg/l), ornithine (1 mM), glycerol (5 mM), and proline (260 mM) (14). Proline (30 mg/l) was added because it is required for DNA synthesis (19). Aspartic acid was not included because it is one of the products of ornithine metabolism and a substrate for arginine synthesis. KnockOut serum replacement (Thermo Fisher Scientific, Inc.) was added at a final concentration of 10% and was used instead of fetal calf serum to establish xeno-free, defined conditions. HDI medium was prepared using oncostatin M (20 ng/ml), FPH1 (20 μ M), M50054 (100 mg/l), NEAA (1x), sodium pyruvate (1x), nicotinamide (1.2 mg/ml), proline (30 ng/ml), and L-glutamine (0.3 mg/ml). Proline and nicotinamide are required for proliferation of primary hepatocytes (19,20).

Conventional media. 201B7 cells were harvested with Accutase, and transferred to a 15 ml tube (Asahi Glass, Tokyo, Japan). The cells were centrifuged at 100 x g for 3 min at 4°C. The cells were spread onto 6-well plates (Asahi Glass) coated with Matrigel at the density of 10⁶ cells for each well and were cultured in ReproFF, L15, William's E (WE) (Thermo Fisher Scientific, Inc.), or Dulbecco's modified Eagle's medium/Ham's F-12 nutrient mixture (DF12; Sigma-Aldrich; Merck KGaA). L15, WE, and DF12 were supplemented with 1.2 mg/ml nicotinamide, 30 ng/ml proline, and 10% Knockout serum replacement. Nicotinamide and proline were added because they are required for proliferation of primary hepatocytes (19,20). For some experiments, 2DG at a concentration of 0, 0.1, 1 or 10 μ M was added and galactose at a concentration of 900 mg/ml was added, as indicated. The cells were cultured for 7 days and then subjected to RT-qPCR.

RT-qPCR. Total RNA (5 μ g), isolated with Isogen (Nippon Gene Co., Ltd., Tokyo, Japan), was used for first-strand cDNA synthesis with SuperScript III reverse transcriptase and oligo (dT) primers (Thermo Fisher Scientific, Inc.), following the manufacturer's instructions. Total RNA from human fetal liver and adult liver was purchased from Clontech Laboratories, Inc. (Mountainview, CA, USA). qPCR was performed in a volume of 20 μ l for 40 cycles of two steps consisting of a 5 sec denaturation step and a 5 sec annealing-extension step, using Fast SYBR Green Master Mix (Thermo Fisher Scientific, Inc.) and the results were analyzed using the MiniOpticon Real-Time PCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The primer sequences are listed in Table I. *RPL19* was used as an endogenous reference control because it is a constitutively expressed housekeeping gene (21). Gene expression levels were analyzed automatically using the MiniOpticon system based on the 2^{- $\Delta\Delta C_q$} method (22). The relative expression was calculated as the expression level of a specific gene divided by that of *RPL19*.

Statistical analysis. Relative expression levels of genes were analyzed by one-factor analysis of variance using JMP version 5.0J software (SAS Institute, Inc., Cary, NC, USA). Student's t-test was used as a post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

To analyze the effect of culturing iPS cells in different media on the expression levels of liver-specific genes, 201B7 cells were cultured in ReproFF, HSM, or HDI for 2 days. RNA was isolated and subjected to RT-qPCR in order to determine the mRNA expression levels of cytosolic AST (Fig. 1A), mitochondrial AST (Fig. 1B), ALT (Fig. 1C) and glycogen synthase (Fig. 1D). The mRNA expression levels of mitochondrial AST, ALT, and glycogen synthase significantly increased by culture in HSM and HDI compared with ReproFF media (Fig. 1B-D). The expression level of cytosolic AST significantly increased by culture in HDI compared with ReproFF media, but not in HSM media (Fig. 1A). These results suggest that the absence of glucose or arginine and the addition of galactose and ornithine increased the expression levels of liver-specific genes and glycogen synthase. Expression levels of each gene were

Table I. Sequences of primers used in the present study.

Gene	Sequence (5'-3')	Product size (bp)	GenBank accession no.
AFP	F-ACACAAAAAGCCCACTCCAG R-GGTGCATACAGGAAGGGATG	147	NM_001134
Cytosolic AST	F-GATGGAGAAGATCGTGCGGAT R-AATCCGGTCAGCCATTGTCTT	133	M37400
Mitochondrial AST	F-ACATGTAGTGACACAGGGCAG R-CACGAGGAACCTGACACTTCA	192	M22632
ALT	F-GAGCCCACTGTACTTGCTCTT R-TTTCCTGGAGTCAGACTGCC	113	BC018207
GS	F-ATATCCCAGGCCTTCCTCAGT R-CGTGGCTCAGTGAAAATGGTG	164	S70004
RPL19	F-CGAATGCCAGAGAAGGTCAC R-CCATGAGAATCCGCTTGTTT	157	BC095445

Primers were designed to have an annealing temperature of 60°C and quantitative polymerase chain reaction was performed for 40 cycles. F, forward; R, reverse; AFP, α -fetoprotein; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GS, glycogen synthase; RPL19, ribosomal protein L19.

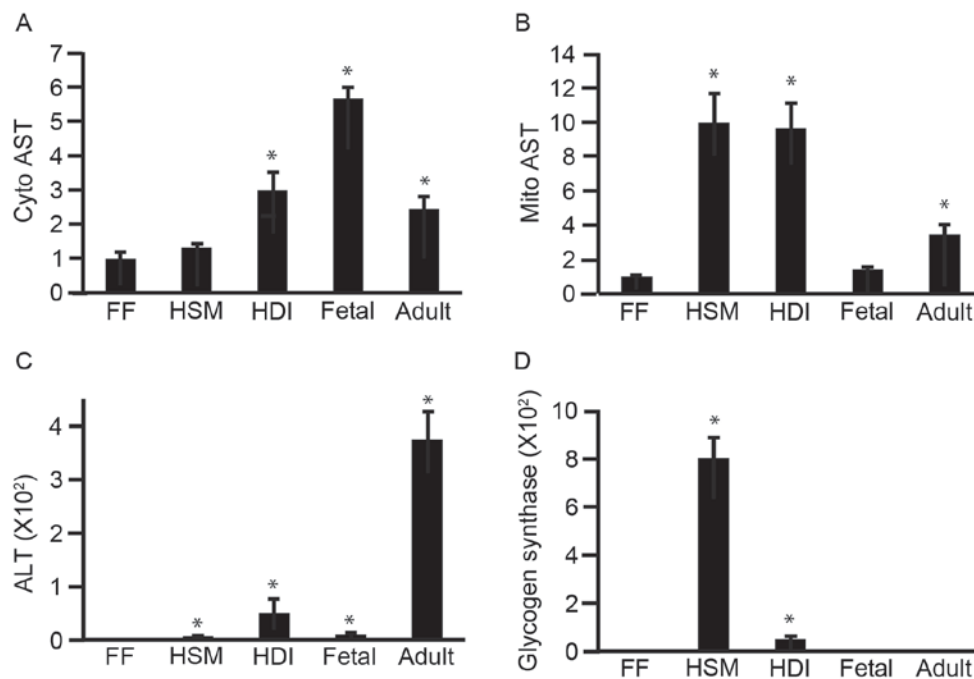


Figure 1. Effect of different culture media on the expression of liver-specific genes. 201B7 cells were cultured in FF, HSM, or HDI media for 2 days. The cells were then subjected to reverse transcription-quantitative polymerase chain reaction to analyze the mRNA expression levels of (A) Cyto AST, (B) Mito AST, (C) ALT and (D) glycogen synthase. *P<0.05 compared with FF (n=3). FF, ReproFF; HSM, hepatocyte selection medium; HDI, hepatocyte differentiation inducer; Cyto, cytosolic; AST, aspartate aminotransferase; Mito, mitochondrial; ALT, alanine aminotransferase; Fetal, fetal liver total RNA; Adult, adult liver total RNA.

analyzed with fetal and adult liver to compare with those of HSM and HDI and identify whether iPS cells acquired expression of liver-specific genes. Cytosolic and mitochondrial AST were expressed in HSM and HDI at comparable levels to fetal and adult liver.

To address the possibility that galactose affected hepatocyte differentiation of 201B7 cells, the cells were cultured in L15, WE, and DF12 media in the absence or presence of galactose

and then total RNA was isolated and subjected to RT-qPCR (Fig. 2). The mRNA expression levels of AFP, a marker of immature hepatocytes, were significantly higher in the L15, WE and DF12 media compared with the ReproFF media (P<0.05; Fig. 2). However, for each medium, mRNA expression levels of AFP were similar in the presence or absence of galactose (Fig. 2). These results suggest that galactose had no effect on the differentiation of 201B7 cells to the hepatocyte lineage.

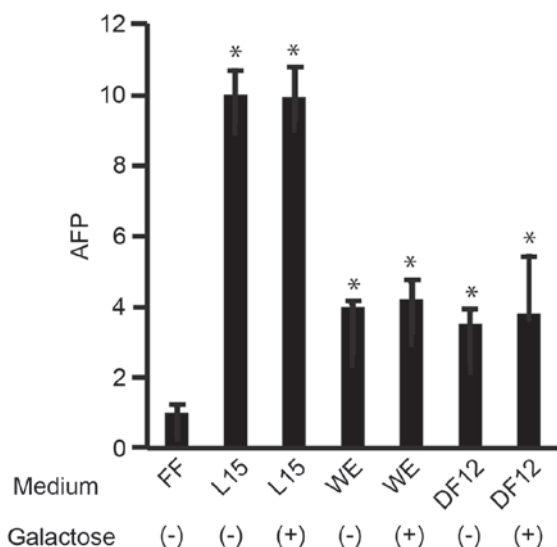


Figure 2. Effect of galactose on expression of the AFP hepatocyte marker. 201B7 cells were cultured in FF, L15, WE, or DF12 media with (+) or without (-) galactose (900 mg/ml). The cells were then subjected to reverse transcription-quantitative polymerase chain reaction to analyze the mRNA expression levels of AFP. * $P < 0.05$ compared with FF ($n = 3$). AFP, α -fetoprotein; FF, ReproFF; L15, Leibovitz's-15; WE, William's E; DF12, Dulbecco's modified Eagle's medium/Ham's F-12 nutrient mixture.

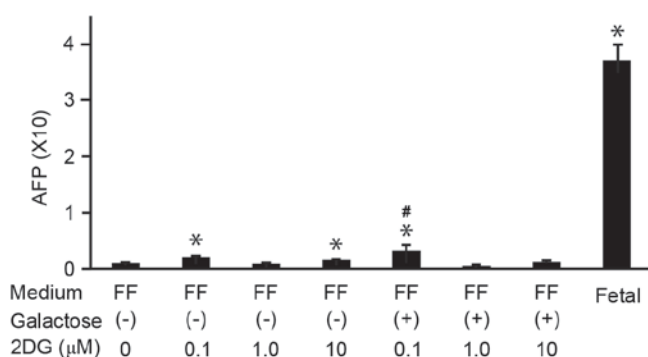


Figure 3. Effects of 2DG and galactose on expression of the AFP hepatocyte marker. 201B7 cells were cultured in FF medium with (+) or without (-) galactose (900 mg/ml) and/or 2DG (0.1-10 μ M). The cells were then subjected to reverse transcription-quantitative polymerase chain reaction to analyze the expression level of AFP. * $P < 0.05$ compared with FF and # $P < 0.05$ compared with 0.1 μ M 2DG ($n = 3$). 2DG, 2-Deoxy-D-glucose; AFP, α -fetoprotein; Fetal, fetal liver total RNA.

To analyze the effect of the glucose analogue 2DG on hepatocyte differentiation of 201B7 cells, the cells were cultured in ReproFF medium in the absence or presence of 2DG and/or galactose. Total RNA was then isolated and subjected to RT-qPCR to determine the mRNA expression levels of AFP (Fig. 3). The mRNA expression levels of AFP significantly increased with the addition of 0.1 μ M 2DG (Fig. 3). In the absence of galactose, AFP was upregulated with 0.1 and 10 μ M but not with 1.0 μ M of 2DG. It was not clear why expression level of AFP with 1.0 μ M of 2DG did not increase. The expression levels of AFP increased at 0.1 mM of 2DG with galactose more than without it. Again, the reason was not clear, but it may be speculated that mechanism of galactose metabolism is involved with hepatocyte

differentiation. Addition of galactose acted synergistically with the 0.1 μ M 2DG treatment to further increase mRNA expression of AFP in 201B7 cells (Fig. 3). These results suggested that 2DG and galactose promoted hepatocyte differentiation of 201B7 cells.

Discussion

Glucose is an indispensable component for cell survival. During somatic cell reprogramming, energy production shifts from an oxidative state to a glycolytic state (23). The source of energy depends on glycolysis in iPS cells (24). Galactose is metabolized through glycolysis following conversion to galactose-1-phosphate by galactokinase. iPS cells do not survive in either HSM or HDI media. In the present study, culturing the iPS cell line 201B7 in either HSM or HDI media resulted in an increase of the expression levels of AFP, suggesting the initiation of iPS cell differentiation to the hepatocyte lineage (15,25). AST, ALT, and glycogen synthase are markers of hepatic function (26). In the present study, the expression levels of AST, ALT, and glycogen synthase increased by culture of 201B7 cells in HSM and HDI media, confirming that hepatocyte differentiation was initiated in iPS cells by these media. Tomizawa *et al* and Phillips *et al* (25,26) have established a medium based on L15 that lacks glucose and contains galactose, that promotes the differentiation of iPS cells to the hepatocyte lineage. These previous reports, together with the present results, suggest that hepatocyte differentiation is initiated in iPS cells cultured in media lacking glucose and supplemented with galactose. The detailed mechanism of this, however, remains unclear.

Next, the aim was to identify whether glucose deprivation or galactose supplementation was responsible for the initiation of hepatocyte differentiation of iPS cells. The present results demonstrated that galactose supplementation did not affect the expression level of AFP. This suggests that galactose supplementation was not involved in hepatocyte differentiation of iPS cells.

A major problem with HSM and HDI media is that iPS cells do not survive for >3 and >7 days, respectively. To overcome this limitation, 2DG was used instead of glucose deprivation. The results demonstrated that 2DG supplementation in the media resulted in a significant increase in the mRNA expression levels of AFP in 201B7 cells. Moreover, galactose supplementation enhanced the 2DG-initiated AFP upregulation. These data suggest that the addition of 2DG and galactose in the media initiated hepatocyte differentiation of iPS cells.

A limitation of the present study was that analysis of hepatocyte differentiation was mainly based on determination of mRNA expression of only one hepatocyte marker, namely AFP. Further studies with immunostaining for hepatocyte-specific markers, such as AFP and albumin, and with functional analysis such as indocyanine green uptake, will be required in the future to further confirm the present results.

In conclusion, the present study demonstrated that hepatocyte differentiation was initiated in iPS cells by culturing in HSM and HDI media. The present results suggest that 2DG may be used as a supplement instead of glucose deprivation to initiate hepatocyte differentiation in iPS cells.

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