Lactate promotes survival and hepatocyte differentiation of human induced pluripotent stem cells in a medium without glucose and supplemented with galactose

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Abstract. Human induced pluripotent stem (iPS) cells initiate hepatocyte differentiation in a medium without glucose and supplemented with galactose, oncostatin M and small molecules [hepatocyte differentiation inducer (HDI)]. To clarify the metabolic differences between iPS cells in HDI and ReproFF (undifferentiated state), a metabolome analysis was performed. iPS cells were cultured in a medium without glucose and supplemented with galactose, as well as 1 mM of calcium lactate, sodium lactate or lactic acid. After 7 days of culture, the cells were subjected to reverse transcription-quantitative PCR analysis. The galactose-1-phosphate concentration was significantly higher in cells cultured in HDI than in those cultured with ReproFF. The lactate concentration in the HDI group was significantly lower than that in the ReproFF group. The expression levels of α-feto protein and albumin were significantly higher in the groups cultured with calcium lactate, sodium lactate and lactic acid as compared with ReproFF. It was suggested that lactate promoted the survival of iPS cells cultured in a medium without glucose and supplemented with galactose. Under these conditions, iPS cells begin to differentiate into a hepatocyte lineage. Lactate may be applied to produce hepatocytes from iPS cells more efficiently.

Introduction

Human induced pluripotent stem (iPS) cells can differentiate into somatic cells (1). Hepatocytes derived from iPS cells are suitable for transplantation into patients with liver insufficiency and for use in toxicity tests (2). These methods have been previously used to obtain hepatocytes from iPS cells.

Protocols for hepatocyte differentiation from iPS cells have been studied. One such approach involves the use of growth factors (3-6). Another method involves the introduction of transcription factors (6-8). Human liver organoids are formed from the assembly of hepatocyte-like cells differentiated from iPS cells, human umbilical vascular endothelial cells and mesenchymal stem cells (9). Human liver organoids are expected to be used as a 'mini-liver' instead of resected liver fragments (10). However, hepatocytes, including organoids, remain immature following the above protocols (11,12). Therefore, methods for obtaining hepatocytes from iPS cells should be further investigated.

Glucose is essential for cell survival and is metabolized to pyruvate (13). Pyruvate enters the tricarboxylic acid cycle to generate energy. Under low-oxygen conditions, pyruvate does not enter the tricarboxylic acid cycle, but is instead metabolized to lactate. Cancer cells continue to produce lactate via glycolysis when oxygen is abundant (the Warburg effect) (14). Human embryonic stem cells also exhibit the Warburg effect (15).

Cells die in glucose-deprived media (16). Under glucose-free conditions, galactose is converted to galactose-1-phosphate and used as an energy source (17). This cycle involves gluconeogenesis and is performed solely by hepatocytes. A Hepatocyte selection medium (HSM) was developed to enrich hepatocytes from co-cultures with iPS cells (18). The HSM does not contain glucose, and galactose is added. In the HSM, iPS cells died within three days; however, hepatocytes survived. After two days of culture in HSM, the expression levels of α-fetoprotein (AFP) and albumin, which are hepatocyte markers, were upregulated, suggesting that hepatocyte differentiation was initiated (19). It was hypothesized that the addition of glucose and galactose promoted hepatocyte differentiation of iPS cell. The HSM was modified into a hepatocyte differentiation inducer (HDI). HDI is based on an HSM with the addition of oncostatin M and an apoptosis inhibitor (20). iPS cells showing elevated expressions survived for 7 days, but eventually died.

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It was hypothesized that iPS cells would differentiate into hepatocytes in a medium without glucose for longer periods. Metabolic changes were focused in search for a novel approach for iPS cells to survive under glucose deprivation. Metabolic alterations need to be clarified to overcome the short-term survival of iPS cells in HDI. Therefore, a metabolome analysis was performed.

Materials and methods

Cell culture. A human iPS cell line, 201B7 (Riken Cell Bank), was cultured on 10 cm dishes (Asahi Glass Co., Ltd.), six-well plates (Asahi Glass Co., Ltd.) or 96-well plates (Asahi Glass Co., Ltd.) coated with Matrigel® (Corning, Inc.) in ReproFF (ReproCell) at 37˚C with 5% carbon dioxide in a humidified chamber. When cells reached confluence, they were rinsed with PBS and harvested using Acutase (Innovative Cell Technologies). The cultured cells were observed under a microscope (CKX41N-31PHP; Olympus Corp.).

Reagents. Calcium lactate was purchased from Nacalai Tesque Inc. Sodium lactate was obtained from Sigma-Aldrich (Merck KGaA). Lactic acid was purchased from Kozakai Pharmaceutical Co., Ltd.

Metabolome analysis. The metabolome analysis was performed by Human Metabolome Technologies. Cells were cultured in 10-cm dishes at confluency. The cells were processed according to the manufacturer's instructions. In brief, culture medium was aspirated and cells were rinsed with 10 ml of 5% mannitol solution (Wako Pure Chemical Industries, Ltd.) in water. The rinse was repeated with 2 ml of 5% mannitol solution. After aspiration of 5% mannitol solution, the entire dish was covered with 800 μl of 100% methanol (Wako Pure Chemical Industries, Ltd.) and left for 30 sec. Subsequently, 550 μl of Internal Standard Solution (Human Metabolome Technologies) was added and slowly pipetted up and down three times. The dishes were then incubated for 30 sec at room temperature. A total of 1,000 μl was transferred from the total 1,350 μl of the supernatant to a 1.5 ml tube and kept on ice. The transferred samples were centrifuged at 1,300 x g at 4˚C for 5 min. Subsequently, 350 μl of the supernatant was transferred to a tube with a filter cup supplied by the company and centrifuged at 1,100 x g, at 4˚C for 2 h. The filter cup was removed and the tube containing the sample was sealed and frozen at 80˚C. The samples were packed on dry ice, sent to Human Metabolome Technologies and subjected to C-SCOPE, which measures metabolites related with energy metabolism.

Cell proliferation assay. After 72 h, an MTS assay (Promega Corporation) was performed according to the manufacturer's instructions. MTS was bio-reduced by the cells into a colored formazan product with reduced absorbance at 490 nm. The absorbance was analyzed at a wavelength of 490 nm with an iMark microplate reader (Bio-Rad Laboratories, Inc.).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA (5 mg), isolated with Isogen (Nippon Gene Co., Ltd.), was used for first-strand cDNA synthesis with SuperScript III reverse transcriptase and oligo (dT) primers (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Real-time qPCR was performed using Fast SYBR Green Master Mix (Thermo Fisher Scientific, Inc.) and the results were analyzed using a Mini Opticon system (Bio-Rad Laboratories, Inc.). qPCR was performed for 40 cycles, with 5 sec for denaturation (95˚C) and 5 sec for annealing-extension (60˚C), using an MJ Mini Cycler (Bio-Rad Laboratories, Inc.). The primer sequences are listed in Table I. Ribosomal protein L19 (RPL19) was used as an endogenous control to monitor the amount of mRNA because it is a constitutively expressed house-keeping gene (21). Gene expression levels were automatically analyzed using the Mini Opticon system based on the $2^{-\Delta\Delta Cq}$ method (22). The relative expression level of a gene was calculated as the gene expression level divided by that of RPL19.

Statistical analysis. Continuous variables are expressed as the mean ± standard deviation. One-way analysis of variance was performed using JMP 10.0.2 software (SAS Institute, Inc.). Tukey's test was used as the post-hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Metabolome analysis. To analyze the differences in the metabolite concentrations between the undifferentiated state and that of cells cultured in HDI, metabolome analysis was performed. iPS cells were cultured on 10-cm dishes in HDI for 2 days and the samples were subjected to metabolome analysis. Among the metabolites, galactose-1-phosphate was applied in order to confirm that galactose entered gluconeogenesis. Lactate was also focused on because it is a typical glycolytic metabolite (23). The galactose-1-phosphate concentration, as measured by Human Metabolome Technologies, was $5.2\pm1.6x10^2$ pmol/10^6 cells in HDI and $0.5\pm0.0x10^2$ pmol/10^6 cells in ReproFF (Fig. 1A). The lactate concentration, as measured by Human Metabolome Technologies, was $2.1\pm0.4x10^3$ pmol/10^6 cells in HDI and $8.7\pm0.3x10^4$ pmol/10^6 cells in ReproFF (Fig. 1B). As expected, galactose in HDI was metabolized to galactose-1-phosphate. Unexpectedly, the lactate concentration was significantly lower in cells cultured in HDI than in those cultured in ReproFF. Lower lactate levels were speculated to be related to limited survival.

Cell proliferation. To analyze whether lactate prolongs iPS cell survival in HSM, a cell proliferation assay was performed. Three types of lactate were used: Calcium lactate, sodium lactate and lactic acid. iPS cells were cultured in HSM supplemented with calcium lactate (Fig. 2A), sodium lactate (Fig. 2B) or lactic acid (Fig. 2C) at concentrations of 0, 3, 10, 100 or 300 μM, and 1, 3, 10, 30 or 100 mM. After 72 h of culture, cells were subjected to a cell proliferation assay. Cell proliferation was 92±13, 87±7 and 52±14% in comparison to ReproFF, at 10, 3 and 1 mM of calcium lacatate, respectively. Cells cultured in calcium lactate showed the highest proliferation potential compared to those cultured in sodium lactate and lactic acid.

Precpitation. In the cell proliferation assay, cultured cells were observed under a microscope. Precipitation was found at the bottom of the dishes after 72 h of culture in HSM

Table I. Primers used for qPCR.

GenBank ID/description	Primer name	Sequence (5' to 3')	Product size, bp
NM_001134			147
qPCR, hAFP, forward	OMC317	ACACAAAAAGCCCACTCCAG	
qPCR, hAFP, reverse	OMC318	GGTGCATACAGGAAGGGATG	
BC000530			157
qPCR, hRPL19, forward	OMC321	CGAATGCCAGAGAAGGTCAC	
qPCR, hRPL19, reverse	OMC322	CCATGAGAATCCGCTTGTTT	
NM_000477			114
qPCR, hAlbumin, forward	OMC329	GCTCGTGAAACACAAGCCCAAG	
qPCR, hAlbumin, reverse	OMC330	GCAAAGCAGGTCTCCTTATCGTC	

AFP, α-fetoprotein; RPL19, ribosomal protein L19; qPCR, quantitative PCR.

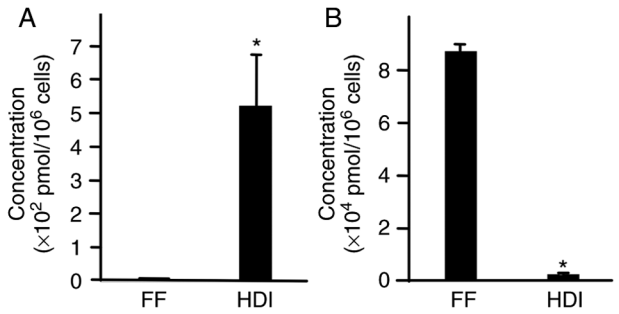

Figure 1. Metabolome analysis. Metabolome analysis was performed to reveal differences in iPS cells cultured in (A) FF or (B) HDI. Values are expressed as the mean ± standard deviation (n=3). *P<0.05 vs. FF. FF, ReproFF; HDI, hepatocyte differentiation inducer.

supplemented with >3 mM of calcium lactate. The medium was transferred to another dish and cultured, and no bacteria or fungi grew. iPS cells were cultured in HSM supplemented with calcium lactate at 0 mM (Fig. 3A), 1 mM (Fig. 3B), 3 mM (Fig. 3C) and 10 mM (Fig. 3D). Precipitation was observed at concentrations of 3 and 10 mM. The precipitation was denser at 10 than at 3 mM. It was speculated that precipitation was formed with calcium from calcium lactate and carbonate in the medium because calcium carbonate is insoluble (24). Combined with the results of the cell proliferation assay, these results suggested that 1 mM of calcium lactate was suitable for further experiments.

Survival with calcium lactate. To observe iPS cell survival, they were cultured in HSM supplemented with calcium lactate (Fig. 4A), sodium lactate (Fig. 4B) or lactic acid (Fig. 4C). After 7 days of culture, the cultured cells had survived. The floating cells were dead cells or debris because the medium did not contain enough glucose.

RT-qPCR. To clarify the differentiation into the hepatocyte lineage, RT-qPCR analysis of specific markers was performed. iPS cells were cultured in HSM supplemented with 1 mM of calcium lactate. After 7 days, RNA was isolated and subjected to qPCR. In the presence of calcium lactate, sodium lactate

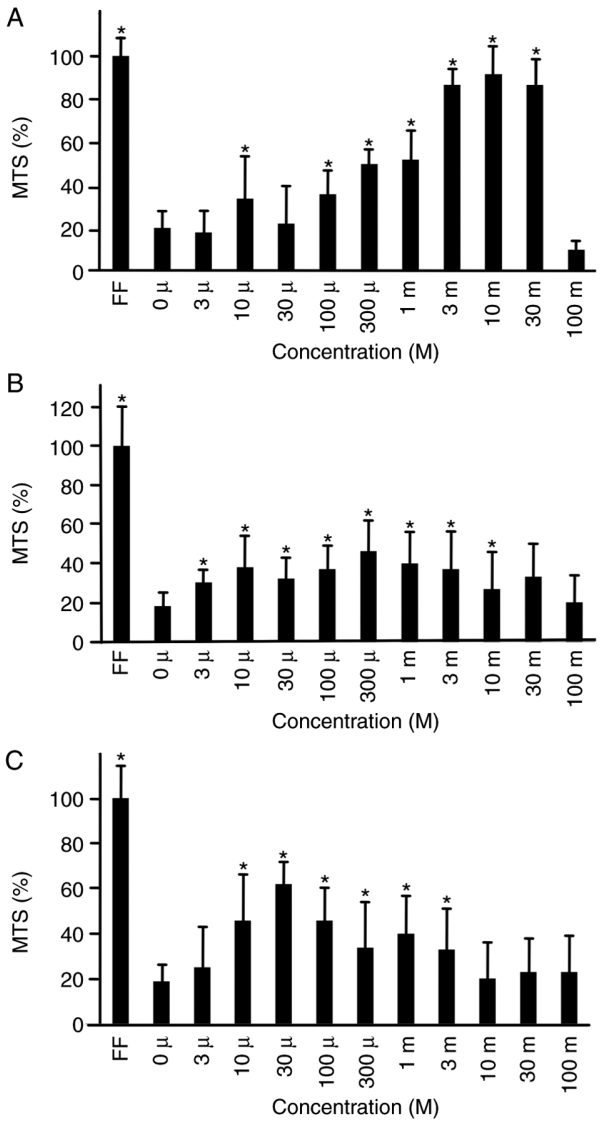

Figure 2. Cell proliferation assay. iPS cells were cultured in a hepatocyte selection medium supplemented with (A) calcium lactate, (B) sodium lactate or (C) lactic acid. After 72 h of culture, cells were subjected to an MTS cell proliferation assay. X-axis: Concentrations of sodium lactate, calcium lactate or lactic acid. Data were normalized to those of ReproFF. Values are expressed as the mean ± standard deviation (n=3). *P<0.05 vs. 0 μm FF, ReproFF.

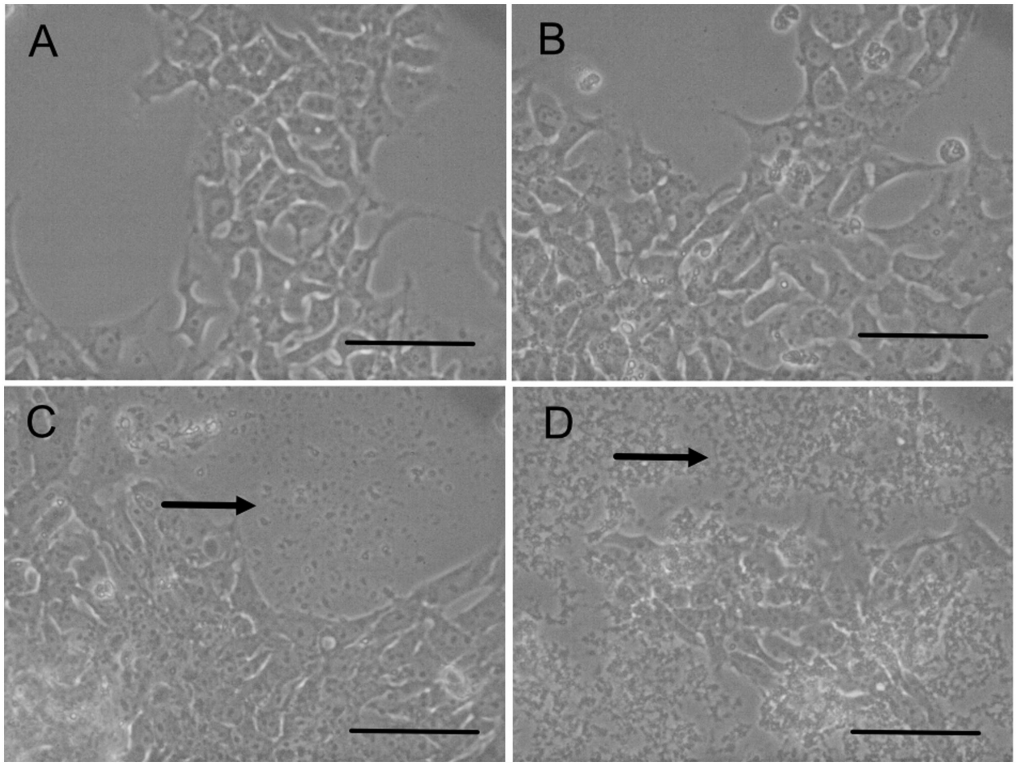

Figure 3. Precipitation. iPS cells were cultured in HSM supplemented with calcium lactate at (A) 0 mM, (B) 1 mM, (C) 3 mM and (D) 10 mM. Precipitation was found on the bottom of the dishes after 24 h of culture at 3 and 10 mM (arrow). Precipitation was denser at 10 than at 3 mM (magnification, x400; scale bars, 100 μm).

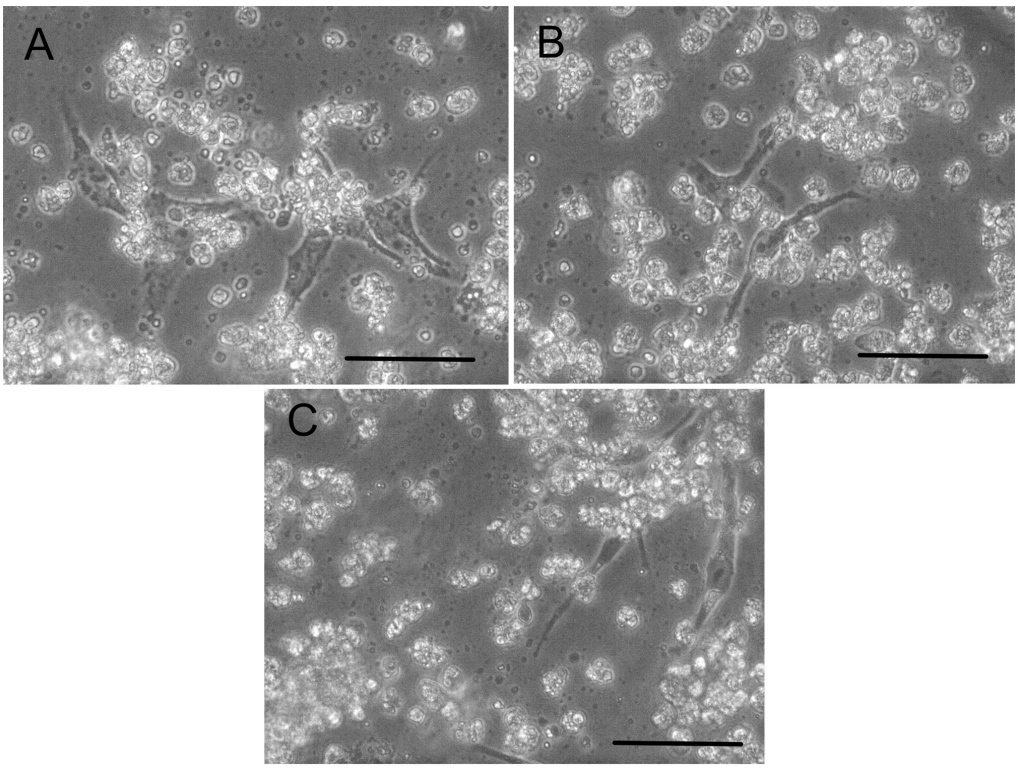

Figure 4. Morphology. Induced pluripotent stem cells were cultured in hepatocyte selection medium with (A) calcium lactate, (B) sodium lactate or (C) lactic acid at 1 mM for 7 days. The cultured cells were observed under a microscope (magnification, x400; scale bar, 100 μm).

and lactic acid, relative AFP expression levels were 5.7±1.4, 3.2±0.3 and 5.8±4.0, respectively (Fig. 5A). In the presence of calcium lactate, sodium lactate and lactic acid, relative albumin expression levels were 30.0±7.5, 6.3±1.8, and 25.0±9.0,

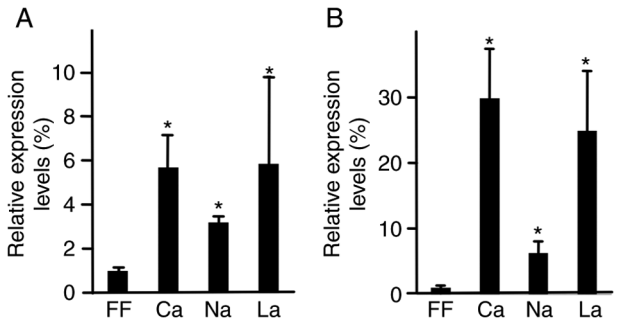

Figure 5. RT-qPCR analysis. Induced pluripotent stem cells were cultured in hepatocyte selection medium with calcium lactate, sodium lactate or lactate for 7 days and subjected to RT-qPCR to measure the expression levels of (A) α-fetoprotein and (B) albumin. Values are expressed as the mean ± standard deviation (n=3). *P<0.05 vs. FF. FF, ReproFF; Ca, calcium lactate; Na, sodium lactate; La, lactic acid; RT-qPCR, reverse transcription-quantitative PCR.

respectively. AFP and albumin expression levels were higher in the calcium lactate, sodium lactate and lactic acid groups than in the ReproFF group (P<0.05).

Discussion

iPS cells die within 3 d in HSM because the medium is deprived of glucose (18). In the current study, the cells survived for >7 d in HSM supplemented with lactate. It has been suggested that iPS cell survival was promoted by lactate. The present metabolome analysis showed that lactate was produced by iPS cells in conventional media, which is consistent with the Warburg effect (15). Galactose is metabolized to galactose-1-phosphate, which then enters glycolysis (17). The present data clearly showed that galactose-1-phosphate was produced in iPS cells from galactose in the HDI medium. The lactate concentration was low in iPS cells cultured in HDI medium. The reason for this is unknown; however, one speculation is that gluconeogenesis was immature. It may be hypothesized that the addition of lactate to HSM would promote iPS cell survival. In a previous study, high lactate levels (up to 28 mM) decreased cell proliferation of human embryonic stem cells (25). This discrepancy was due to the concentration of glucose. In the present study, HSM deprived of glucose was used. Odenwelder *et al* (26) reported that ^{13}C-labeled lactate enters the tricarboxylic acid cycle via pyruvate. In this study, lactate was converted to pyruvate and used as the energy substrate (27). This metabolic pathway is called the Cori cycle and is executed solely by hepatocytes (27). In addition to survival, lactate promotes cell proliferation (23).

The present RT-qPCR results indicated that AFP and albumin levels were upregulated in cells in HSM with calcium lactate. It was suggested that hepatocyte differentiation was promoted by HSM with lactate, as these two genes are hepatocyte markers (28,29). Previous studies by our group showed that iPS differentiation toward the hepatocyte lineage was promoted in medium without glucose and supplemented with galactose (18,19). Hepatocyte differentiation is promoted by inhibiting glycolysis with 3-bromopyruvate and 2-deoxy-d-glucose, pyruvate and glucose analogs, respectively (29). The current data are consistent with those of the previous studies by our group. One major issue with HSM and HDI is that the cultured cells do not survive long enough to differentiate into hepatocytes. It may be possible to promote the differentiation of iPS cells into hepatocytes if the cells survive for longer. In the present study, cultured cells survived for >7 d and exhibited an upregulation of hepatocyte markers. The previous studies by our group and the current study indicate that hepatocyte differentiation of iPS cells is promoted in media without glucose and supplemented with galactose. Lactate may promote the differentiation of iPS cells to hepatocytes, while the Cori cycle is activated in hepatocytes. Sinton *et al* (30) reported that steatosis is induced in hepatocyte-like cells differentiated from iPS cells when added with lactate, pyruvate and octanoate. Hepatocyte differentiated from iPS cells would be useful for research on liver diseases.

Lactate was added to the HSM and galactose was added to the medium without glucose. Thus, it is possible that lactate affects iPS cell differentiation. Human embryonic stem cells show decreased pluripotency in media containing 11 mM of lactate (25). It is possible that lactate promotes hepatocyte differentiation; however, this remains to be further clarified.

One major limitation of the present study was that endodermal and hepatocyte markers other than AFP and albumin were not analyzed. It is not known what role the obtained cells had in the differentiation toward a hepatocyte lineage.

Our next step would be to determine the differentiation state of cells cultured in HSM supplemented with lactate. In the future, metabolome analysis will be performed using lactate labeled with carbon 13.

In conclusion, lactate promoted the survival of iPS cells cultured in a medium without glucose and supplemented with galactose. Under these conditions, iPS cells began differentiating into the hepatocyte lineage. Lactate may be a novel approach to produce hepatocytes from iPS cells.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MT performed the experiments and wrote the manuscript. FS and TM performed the statistical analysis. HT and MS performed the MTS assay and took photographs of cells. All authors confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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