39˚C-*in vitro* culture facilitates hepatic functions of hepatocyte-like cells derived from human embryonic stem cells

Running Head: Hepatic functions facilitated by 39˚C culture

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Abstract

Hepatocyte-like cells derived from human pluripotent stem cells (hPSC-HLCs) may offer an alternative to primary hepatocytes that are commonly used for drug screenings and toxicity tests. Although tremendous efforts have been made to facilitate hepatic functions of hPSC-HLCs using growth factors and chemicals, these cells have not yet reached hepatic functions that are comparable to primary hepatocytes. Therefore, there exists a critical need to use an alternative trigger to facilitate hepatic functions in hPSC-HLCs. We noted that human liver temperature (around 39˚C) is higher than normal human body temperature (around 36.5˚C), yet hepatocytes are generally cultured at 37˚C. Here, we tested the hypothesis that hepatic functions of hPSC-HLCs would be facilitated under physiologically-relevant hepatic temperatures. We identified the optimal temperature by treating HLCs derived from H9 human embryonic stem cells (hESC-HLCs) at 39˚C and 42˚C. As expected, 42˚C caused significantly greater cell death compared to 39˚C. Next, we measured the activities of cytochrome P450 3A4 (CYP3A4), which is one of the most important enzymes for hepatic detoxification. The 39˚C-treated hESC-HLCs had CYP3A4 activities that were greater than the 37˚C-treated hESC-HLCs. Albumin secretion significantly increased in the 39˚C-treated hESC-HLCs. In combination with existing hepatic differentiation protocols, the method proposed here may further improve hepatic functions for hPSCs and thereby aid drug discovery efforts and improve drug toxicity tests.

Introduction

Hepatocytes are the major cellular component of liver tissue and play an important role in protein synthesis/storage, carbohydrate metabolism, and production of cholesterol, bile acids, and phospholipids that contribute to homeostasis in vertebrates. The evaluation of the safety of drug candidates often focuses on vertebrate livers because hepatocytes metabolize chemical substances and drugs *in vivo* [1]. For drug screening and toxicological assays, human primary hepatocytes (hPH) are commonly used, but these cells are very difficult to obtain from healthy donors and often lose their functions when *in vitro*. Furthermore, hPH from donors are highly variable in cell functionality due to differences in purification, culture, and assay protocols, resulting in inconsistency among results. Hepatic cell lines (e.g., HepG2 and HepaRG) have been used as alternatives to hPH [2] because they can be easily obtained and expanded. However, these cells have extremely low drug metabolism, and transportation functions compared to hPH and do not represent healthy hepatic functions due to their cancerous characteristics [3]. In response to the need for alternative cost-efficient, homogenous, readily available, and *in vitro*-viable cells for drug screening and toxicological tests, researchers have recently turned to stem cell technologies.

Hepatocyte-like cells derived from human pluripotent stem cells (hPSC-HLC) have considerable potential to provide optimal hepatocyte function during drug screening and toxicity tests [4]. Human pluripotent stem cells (hPSCs), embryonic stem cells (ESCs) [5],

and induced pluripotent stem cells (iPSCs) [6] all can differentiate into almost any kind of cell and possess the ability to self-renew indefinitely. Previous researchers have successfully used growth factors and other chemicals to induce differentiation of hPSCs to hPSC-HLCs [7-11], but these studies did not achieve mature hepatic function of hPSC-HLCs *in vitro*. The results of these studies clearly demonstrate the need to develop new methods to obtain *in vitro* hepatic function of hPSC-HLCs.

Previous research investigating methods to achieve mature hepatic function of hPSC-HLCs *in vitro* have not fully considered the *in vivo* physiological conditions of a human liver. The temperature in a human liver is around 39˚C, which is higher than the average human body temperature of 36.5˚C. The high level of metabolic activity in the liver is either the cause or the consequence of this temperature that is higher than the surrounding tissues. Nevertheless, most *in vitro* cultures of hepatocytes are performed at 37˚C, which is approximating average body temperature. On the other hands, 42˚C is stressful for cells, and cause cell death and detachment in some type of cells [12-14]. We hypothesized that *in vivo* physiological temperature is required to express hepatic functions in hPSC-HLCs.

Here, we developed a method to promote hepatic functions using a temperature that was higher than the conventional cell culture setting of 37˚C (**Figure 1**). To test this hypothesis, we optimized the various temperatures for functionalizing HLCs derived from hESC (hESC-HLCs) by reducing cell damage caused by excessive heat treatments. We

validated our proposed method by evaluating cytochrome P450 3A4 (CYP3A4) activity, albumin secretion, glycogen storage, drug uptake/excretion, and the expression of proteins associated with hepatocyte maturations.

Materials and Methods

hPSC culture. hESCs were used according to the guidelines provided by the ethical committee of Kyoto University. H9 hESCs used in this study were purchased from WiCell Research Institute (Madison, WI, USA). Prior to culturing, hESC-certified Matrigel (Corning, Corning, NY, USA) was diluted with Dulbecco's modified Eagle medium (DMEM)/F12 medium (Sigma-Aldrich, St. Louis, MO, USA) at a 1:75 (v/v) ratio and coated onto a culture dish. The Matrigel was incubated in the culture dish for 24h at 4˚C. Then, excess Matrigel was removed, and the coated dish was washed with fresh DMEM/F12 medium.

We used mTeSR-1-defined medium (Stem Cell Technologies, Vancouver, Canada) for daily culturing of hPSCs. For passaging, cells were dissociated with TryPLE Express (Thermo Fisher Scientific, Tokyo, Japan) for 3 min at 37˚C and then harvested. A cell strainer was used to remove undesired cell aggregates from the cell suspension, and cells were then centrifuged at 200 × *g* for 3 min and resuspended in mTeSR-1 medium. Live/dead cells were counted using a NucleoCounter NC-200 (Chemetec, Baton Rouge, LA, USA). We used mTeSR-1 medium containing 10 μM of the ROCK inhibitor Y-27632 (Wako, Osaka, Japan) to prevent apoptosis of dissociated hPSCs on day 1. On subsequent days, we used mTeSR-1 medium without the ROCK inhibitor with daily medium changes.

Hepatic differentiation from hPSCs. Prior to inducing differentiation, we coated a cell culture dish with 0.1% gelatin in phosphate-buffered saline (PBS, Thermo Fisher Scientific)

for 30 min at room temperature (25˚C). We then aspirated the gelatin solution and introduced a DMEM/F12 medium (Sigma-Aldrich) onto the culture dish for serum coating at 37˚C for 24 h. The medium was supplemented with 10% (v/v) fetal bovine serum (FBS, Cell Culture Bioscience, Tokyo, Japan), penicillin/streptomycin (Wako), and 100 μM β-mercaptoethanol (Sigma-Aldrich). The coated dish was then rinsed with fresh medium.

To induce endoderm differentiation, cultured hPSCs were washed with PBS and treated with TryPLE Express at 37˚C for 5 min, followed by the addition of basal medium and the transfer of the cell suspension into a 15 mL tube. Cells were centrifuged at 200 × *g* for 3 min, the supernatant was removed, and then the cells were resuspended in mTeSR-1 medium supplemented with 10 μM Y27632 and 100 ng mL^{-1} activin A (R&D Systems, Minneapolis, MN, USA), plated on a serum-coated culture dish, and cultured in a humidified incubator at 37˚C with 5% CO_2 for 24 h. At the end of day 1, the medium was replaced with fresh mTeSR-1 medium supplemented with 10 μM Y27632 and 100 ng mL^{-1} activin A and cultured for another 24 h. On day 2, the medium was replaced with mTeSR-1 medium supplemented with 10 μM Y27632, 100 ng mL^{-1} activin A, 10 ng mL^{-1} BMP-4 (R&D Systems), 10 μM LY294002 (Cayman Chemical, Ann Arbor, MI, USA), and 3 μM CHIR99021 (Stemgent, Cambridge, MA, USA), and cells were incubated for 24 h. On day 3, the medium was replaced with mTeSR-1 medium supplemented with 10 μM Y27632, 100 ng mL^{-1} activin A, 10 ng mL^{-1} BMP-4, and 10 μM LY294002, and cells were incubated for 24 h. On day 4, the medium was replaced with Roswell Park Memorial Institute (RPMI) medium (Thermo Fisher Scientific) supplemented with B-27 (Thermo Fisher Scientific), 100 ng mL^{-1} activin A, and 100 ng mL^{-1} bFGF, and cells were incubated for 24 h.

To induce ADE specification, cells were treated with RPMI medium supplemented with 50 ng mL^{-1} activin A, with daily medium changes for 3 days. Cells were then treated with RPMI

medium supplemented with 20 ng mL^{-1} BMP-4 and 10 ng mL^{-1} FGF-10 (R&D systems), with daily medium changes for 4 days.

On day 12, the medium was replaced with hepatocyte maturation medium (hepatocyte basal medium (Lonza, Basel, Switzerland) supplemented with 30 ng mL^{-1} oncostatin M (R&D Systems) and 50 ng mL^{-1} hepatocyte growth factor (PeproTech, Rocky Hill, NJ) to induce maturation of the differentiated hepatocytes. Cells were incubated at 37, 39, or 42˚C and half the total amount of the medium was changed every day.

Cytochrome P450 3A4 activity assay. We used a cytochrome P450 3A4 (CYP3A4) Assay and Screening System with Luciferin-IPA (Promega, Madison, MI, USA) to assess CYP3A4 activity. Samples were treated with luciferin-IPA substrate (1:1000) in hepatocyte maturation medium at 0, 3, 6, 9, 12, and 15 days after heat treatment (DAH) of either 37˚C or 39˚C. We collected the medium after 1 h and added Luciferin Detection Reagent (Promega). After 15min, the CYP3A4 activity was measured in each sample with a Synergy HTX multi-mode reader. The activity was normalized by the total number of cells.

Immunocytochemistry. Cells were fixed with 4% paraformaldehyde (Wako, 161-20141) in PBS for 20 min at 25˚C and then permeabilized with 0.1% Triton X-100 (MP Biomedicals, CA, USA) in PBS for 10 min at 25˚C. Subsequently, cells were blocked in blocking buffer (5% normal goat serum, Vector; 5% normal donkey serum, Wako; 3% bovine serum albumin, Sigma-Aldrich; and 0.1% Tween-20, Nacalai Tesque, Inc, Kyoto, Japan) in PBS at 4˚C for 16 h and then incubated at 4˚C for 16 h with the primary antibody (anti-human A1AT rabbit IgG, 1:800, Dako, Tokyo, Japan. A0012; anti-human CYP3A4 mouse IgG, 1:25, Santa Cruz Biotechnology, Inc, CA, USA, sc-53850; and anti-human ALB mouse IgG, 1:50, R&D Systems, 188835) in blocking buffer. Cells were then incubated at 37˚C for 60 min with a

secondary antibody (AlexaFluor 488 Donkey anti-rabbit IgG, 1:1000, Jackson ImmunoResearch, PA, USA 711-546-152 and AlexaFluor 647 Donkey anti-mouse IgG, 1:1000, Jackson ImmunoResearch, 715-606-150) in blocking buffer prior to a final incubation with 4′,6-diamidino-2-phenylindole (Wako 342-07431) at 25˚C for 30 min.

ICG uptake/excretion assay. Briefly, 1 mg mL^{-1} ICG (Sigma-Aldrich) was dissolved in the hepatocyte-maturation medium. Cells were treated with the ICG solution for 1 h, rinsed with hepatocyte-maturation medium, and then observed using a bright-field microscope (Olympus, Tokyo, Japan). After 24 h, cells were observed again to visualize excretion capability. To calculate the ICG positive area, we used imageJ software program (National Institutes of Health, Bethesda, MD, USA).

Periodic Acid Schiff (PAS) staining. We used a PAS staining kit for detection of aldehydes and mucosubstances (Merck, Tokyo, Japan). To calculate the ICG positive area, we also used imageJ software program.

Image acquisition. Each sample containing cells was placed on the stage of a Nikon ECLIPSE Ti inverted fluorescence microscope equipped with a CFI plan fluor 10×/0.30 N.A. objective lens (Nikon, Tokyo, Japan), a CCD camera (ORCA-R2; Hamamatsu Photonics, Hamamatsu City, Japan), a mercury lamp (Intensilight; Nikon), an XYZ automated stage (Ti-S-ER motorized stage with encoders; Nikon), and filter cubes for fluorescence channels

(DAPI and GFP HYQ; Nikon). For image acquisition, the exposure times were set at 200 ms for DAPI, 200 ms for GFP HYQ for A1AT, and 800 ms for CYP3A4 and ALB.

Statistical analysis. A two-tailed Student's *t*-test was carried out in Microsoft Excel.

Results

Effect of temperature on cell death

We found that hESC-HLCs that were incubated at 39˚C, the *in vivo* temperature of the human liver, had comparable measures of survival with the currently-used cell culture temperature of 37˚C. To investigate the effect of heat treatment on cellular phenotypes of hESC-HLCs, we improved our differentiation method with single-cell dissociated hPSCs [15] (**Figure 2A**). The hepatic progenitor cells derived from H9 hESCs (hESC-HPC) at day 14 were treated in 37, 39, and 42˚C. While cells treated in 42˚C at 15 DAH showed massive cell death, 39˚C-treated cells did not show an increase in cell death or morphological changes compared with 37˚C-treated cells (**Figure 2A**). Live/Dead cell counting by nucleocounter showed that the proportions of 42˚C-treated cells that were living were significantly reduced (54 ± 12% and 13 ± 12% at 6 and 15 DAH, respectively) compared to that of 39˚C-treated cells (82 ± 13% and 92 ± 8% at 6 and 15 DAH, respectively; **Figure 2B**). As expected, the 42˚C-treatment showed massive cell death compared with the other two treatments. Previous

research has suggested that a strong heat stress can cause apoptosis or shedding in certain cells [13-15]. Therefore, we used our and previous results to further assess the facilitation of hepatic functions only in the 37˚C and 39˚C-trematment for hESC-HLCs.

Effect of heat treatment on at 39˚C facilitates hepatic functions

We found that 39˚C-treated cells demonstrated improved hepatic function compared to the 37˚C-treated cells. During the hepatic maturation process, both 37˚C- and 39˚C-treated cells increased in measures of CYP3A4 activity, but CYP3A4 activity in the 39˚C-treated cells significantly increased from 3 DAH to 15 DAH ($p < 0.05$) and then reached a plateau at 12 DAH earlier than 37˚C-treated cells (**Figure 2C**). CYP3A4 was detected in 37˚C and 39˚C-treated cells at 12 DAH when the activity level was the highest, but there were no significant differences between these measures (**Figure 2D**).

We also examined the effects of heat treatment on other hepatic functions. The 37˚C- and 39˚C-treated cells gradually increased albumin secretion from 3 to 15 DAH, and 39˚C-treated cells showed significantly increased at 9 DAH ($p = 0.002$) (**Figure 2E**). Albumin was detected in 37˚C- and 39˚C-treated cells at 12 DAH, but there were no significant differences in these measures (**Figure 2F**). To visually confirm the ability to uptake and excrete drugs, indocyanine green (ICG) was used for 37˚C- and 39˚C-treated hESC-HLCs (**Figure 2G** and **H**). The ICG uptake level for 39˚C-treated cells significantly increased at 12 DAH ($p = 0.039$), but the excretion level was not significantly different at 13 DAH. The PAS staining revealed

that over 50% of the 37˚C- and 39˚C-treated hESC-HLCs showed cellular glycogen storage (**Figure 2I** and **J**). To confirm the maturity of hESC-HLCs, we conducted immunocytochemistry for one of the maturation markers, α1anti-trypsin (A1AT). A1AT was detected in 37˚C- and 39˚C-treated hESC-HLCs, but there were no significant differences between the two temperature treatments **(Figure 2K)**. These results show that hESC-HLCs incubated at 39˚C show overall improved hepatic functions compared to those incubated at 37˚C.

Discussion

The results of this study show that 39˚C-treatment may facilitate hepatic functions of hESC-HLCs. In particular, we found that CYP3A4 activity was significantly higher in 39˚C-treated hESC-HLCs from 3 DAH to 15 DAH compared with 37˚C-treated hESC-HLCs. CYP3A4 is mainly produced in the mature liver and intestine and is known to be the most important metabolic enzyme to consider when optimizing drug treatments. Functional expression of CYP3A4 is essential for drug screening and toxicological assays using hepatocytes *in vitro*. The results of this study may be used to facilitate *in vitro* hepatocytes that are suitable for CYP3A4 evaluations.

It is possible that CYP3A4 activity is facilitated by a temperature dependent heat shock protein. Previous research has shown that HSP90 is involved in the CYP3A4 synthesis

process as a molecular chaperone [16]. Furthermore, similar to the liver, skeletal muscle and brain tissue also produce heat. Skeletal muscle contains myoblasts while neural stem/progenitor cells are contained in brain tissues [17, 18]. Given the results presented here, researchers should consider culturing cells at temperatures that attempt to replicate the temperatures that are experienced *in vivo*.

Conclusion

Although previous researchers have attempted to promote the hepatic function of hPSC-HLCs, this is the first report showing that the culture temperature of hESC-HLCs improves hepatic function. Previous studies have improved hepatic functions of hPSC-HLCs with the addition of growth and chemical factors [7-11], a stiff scaffold [19], and with stimulation via culture solution flow [20]. During embryonic development, the liver develops adjacent to the heart [21], and the elongation and pressure stimuli caused by the beating heart may contribute to the promotion of hepatic functions. Future research should also investigate the contribution of physical stimuli, in addition to temperature, in promoting hepatic functions.

Researchers and industry scientists could increase the culture temperature to 39˚C to increase the activity of CYP3A4 and albumin secretion. This simple change in protocol could facilitate hepatic functions of hESC-HLCs without impairing hepatic function. Future

research should investigate the mechanisms by which such hepatic functions were improved by heat treatment. These results could also be extended to investigate the effects of 39˚C incubation on other cytochrome P450s and drug metabolizing enzymes. These and other investigations may have significant practical contributions to drug screening and toxicity testing protocols.

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Figure legends

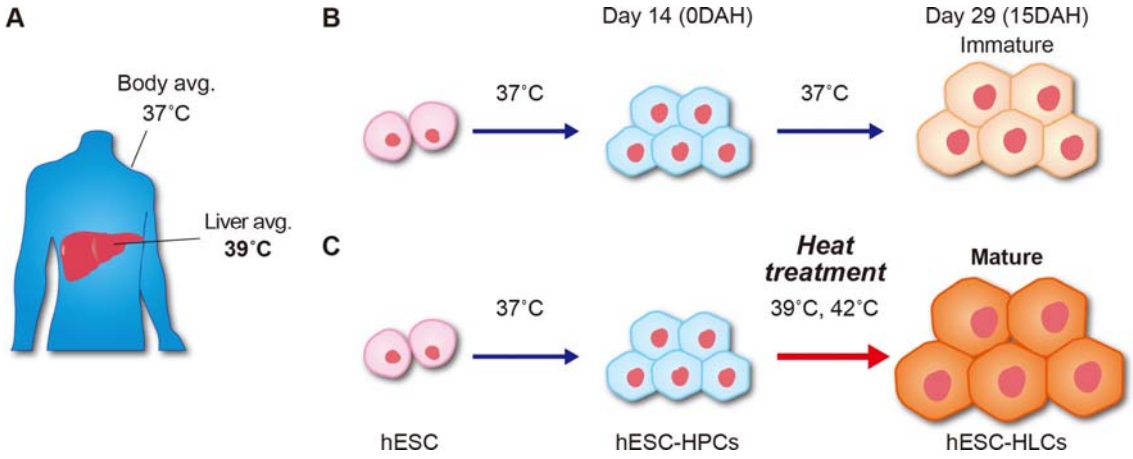

Figure 1. Functionalization of hepatocyte-like cells derived from H9 hESCs (hESC-HLCs) by heat treatment. (A) Average (avg.) temperature of human body and liver. (B) Schematic representation of the protocol to functionalize hESC-HLCs. Days indicate days from initiation of differentiation. DAH treatment. hESC-HPCs; hepatic progenitor cells derived from hESCs. (C) Schematic representation of the protocol for heat treatment to hESC-HLCs.

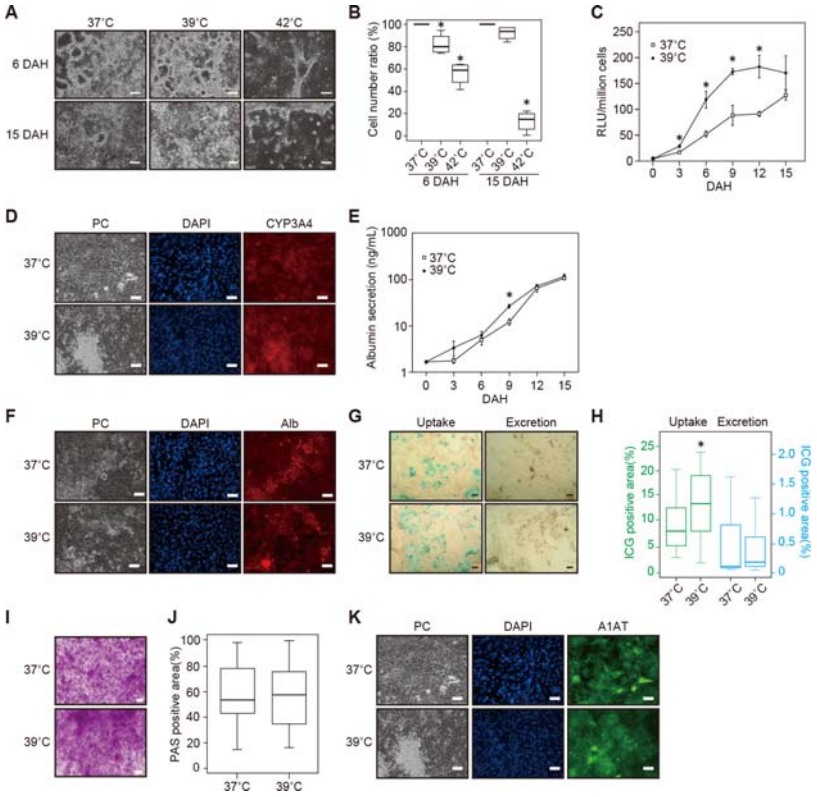

Figure 2. Mild heat treatment facilitates hepatic functions and maturation of hESC-HLCs. In all panels, where applicable, center lines of box plots indicate medians; box limits indicate the 25th and 75th percentiles as determined by *R* software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles; * = $P < 0.05$; each plot and error bar represents a mean ± SD ; and DAPI was used for nuclei staining. (A) Microscopic images of hepatocyte-like cells derived from H9 hESC (hESC-HLCs) treated with 37, 39, and 42˚C for 6 and 15 days. Scale bars = 100 µm. (B) Box plot showing the percentiles of living hESC-HLCs after heat treatments at 37, 39, and 42˚C after 6 and 15 days (n = 4). (C) CYP3A4 activities in hESC-HLCs incubated at 37 and 39˚C (n = 4). (D) Phase contrast and fluorescent micrographs of the expression of CYP3A4 in hESC-HLCs. Scale bars = 50 µm. (E) Albumin secretion from 37˚C- (white squares) and 39˚C-treated hESC-HLCs (circles). * = $P < 0.05$. (n = 4). (F) The expression of albumin in 37˚C- and 39˚C-treated hESC-HLCs at 12 DAH. Scale bars = 50 µm. (G) Micrographs of 37˚C- and 39˚C-treated hESC-HLCs at 12 DAH treated with indocyanine green (ICG). Scale bars = 50 µm. (n = 20). (H) Box plots of the percentiles of cells stained with ICG. (I, J) PAS and quantitative data. (n = 20). (K). The expression of A1AT in 37˚C- and 39˚C-treated hESC-HLCs at 12 DAH. Scale bars = 50 µm.