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ORIGINAL ARTICLE

Direct differentiation of hepatic cells from human induced pluripotent stem cells using a limited number of cytokines

Akemi Takata · Motoyuki Otsuka · Tomomi Kogiso · Kentaro Kojima · Takeshi Yoshikawa · Ryosuke Tateishi · Naoya Kato · Shuichiro Shiina · Haruhiko Yoshida · Masao Omata · Kazuhiko Koike

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Abstract
Purpose Development of improved protocols for differentiating induced pluripotent stem (iPS) cells into hepatic cells is an important step toward their use in the field of hepatology. Specifically, the number of different cytokines should be reduced to limit undesired effects and to reduce the cost of the process. In this report, we describe a simple method for directing human iPS cells to differentiate into hepatic cells using only two cytokines and a short incubation time.
Methods A two-step protocol for differentiating iPS cells into hepatic cells was developed. A high dose of activin A was applied for 3 days to induce definitive endoderm formation. Subsequently, cells were treated with hepatocyte growth factor (HGF) for 5 days to generate hepatic cells. Differentiation was confirmed by immunostaining for differentiation markers. Albumin mRNA levels in differentiated hepatic cells generated using a previously tested three-step protocol that uses activin A, fibroblast growth factor (FGF)/bone morphogenetic protein (BMP), and HGF, and our new protocol were compared to determine the efficiency of differentiation.
Results Our two-step protocol induced the differentiation of iPS cells into hepatic cells and required a shorter differentiation period than the previous three-step protocol. The differentiation efficiencies of the two protocols were comparable and the induced hepatic cells were functional.
Conclusions Developing efficient induction and culture methods to generate more highly matured hepatocytes is essential for regenerative cell-based therapies. Our protocol provides a simple, cost-effective, and time-saving approach for generating hepatic cells from iPS cells.

Keywords iPS · Differentiation · Hepatocyte

Introduction

Induced pluripotent stem (iPS) cells have been generated from mature somatic fibroblasts through over-expression of four transcription factors: Oct-4, Sox2, Klf4, and c-Myc [1]. Since the initial creation of iPS cells, much effort has gone into improving the methods used to generate them, including the use of chemical induction and avoiding or reducing the use of inducing genes [2–5]. Human iPS cells have been shown to resemble human embryonic stem (hES) cells in terms of pluripotency [6]. Indeed, iPS cells are apparently able to proliferate infinitely and differentiate into cell types of various lineages in vitro [7, 8]. Differentiated cells generated from iPS cells derived from patients have great potential in regenerative medicine in tissue replacement and cell-based therapies, and may also help with drug discovery and/or in understanding disease pathogenesis.

To date, human iPS cells have been shown to differentiate into various cell types including cardiovascular cells

A. Takata · M. Otsuka (✉) · K. Kojima · T. Yoshikawa · R. Tateishi · S. Shiina · H. Yoshida · M. Omata · K. Koike
Department of Gastroenterology, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan
e-mail: otsukamo-tky@umin.ac.jp

T. Kogiso
Department of Medicine and Institute of Gastroenterology, Tokyo Women's Medical University, Tokyo, Japan

N. Kato
Global COE Program, Centre for Education and Research for the Advanced Genome-Based Medicine, Unit of Disease Control Genome Medicine, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan

[9], pancreatic cells [10, 11], neural cells [12], and hematopoietic cells [13, 14]. Several studies have also demonstrated the capacity of human iPS cells to differentiate into hepatocyte-like cells [15–17]. Notably, two recent studies reported the first successful differentiation of human iPS cells into endoderm-derived hepatocytes [15, 17]. However, while the protocols used to generate specific cell types from iPS cells have been continually improved, those presently being used still involve the use of multiple undefined and expensive growth factors and/or cytokines and are time-consuming. Thus, the development of simpler approaches for differentiating iPS cells into hepatocytes will be a great advantage for their potential future use.

In protocols used in recent studies to induce the formation of hepatic cells from human iPS cells, activin A has been used to induce endodermal cell formation, and BMP4 and FGF2 then specify hepatic cell formation, and, subsequently, HGF specifies hepatic cell formation [15]. However, because the rationale for using these cytokines is based largely on studies of mouse embryonic development, it remains to be determined whether the use of these cytokines, and the culture conditions are, in fact, optimal for the induction of hepatic cell formation from human iPS cells in vitro. In this study, we report a simpler, cost-effective, and time-saving protocol for driving human iPS cells to differentiate into hepatic cells using only two cytokines.

Methods

Cytokines

Basic fibroblast growth factor (bFGF) was obtained from Invitrogen (Carlsbad, CA). Activin A was acquired from Sigma-Aldrich (St. Louis, MO). HGF, fibroblast growth factor 4 (FGF4), and bone morphogenetic protein 2 (BMP2) were purchased from Wako (Osaka, Japan). Oncostatin M was purchased from R&D Systems (Minneapolis, MN).

Antibodies

A rabbit anti-HNF3β/FOXA2 antibody (#07-633; Millipore, Temecula, CA), a goat anti-SOX17 antibody (MAB1924; R&D systems), a rabbit anti-α-fetoprotein (AFP) antibody (N1501; Dako, Kyoto, Japan), and a goat anti-albumin antibody (A80-129A; Bethyl Laboratories Inc., Montgomery, TX) were used.

Cell culture

STO cells (mouse embryonic fibroblast cell lines) were obtained from the European Collection of Cell Cultures (ECACC; Porton Down, UK) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2 mM glutamine and 10% fetal bovine serum. Cells were treated with mitomycin C (20 μg/mL; Nacalai Tesque, Kyoto, Japan) for 3 h and were plated in 0.1% gelatin-coated 60 or 35 mm dishes (1.5×10^5 cells/mL) the following day to be used as feeder cells for iPS cell culture. Two human iPS cell lines, HPS0001, and HPS0002, which were established by transducing human fibroblasts with lentiviruses carrying Oct-3/4 Sox2 and Klf4, with or without c-Myc, respectively [2, 7], were obtained from the Riken Bioresource Centre (Ibaraki, Japan). iPS cells were seeded onto mitomycin C-treated STO cells and were cultured at 37°C in a standard humidified gas atmosphere containing 5% CO_2 Primate ES medium (ReproCELL, Kanagawa, Japan) supplemented with bFGF (5 ng/mL) was used to maintain iPS cell growth, according to supplier's instructions. The growth medium was replaced daily.

Hepatic differentiation of human iPS cells

To drive iPS cells to differentiate directly into endodermal cells without embryoid body formation, iPS cells were seeded at 70–80% confluence and were incubated in serum-free RPMI 1640 medium (Invitrogen) supplemented with B27 (Invitrogen) and activin A (100 ng/mL) for 3 days. Subsequently, differentiated endodermal cells were treated with HGF (20 ng/mL) for 5 days to generate hepatic cells. To achieve final hepatic cell maturation, cells were cultured for 5 days in hepatocyte culture medium (HCM; Lonza, Walkersville, MD) supplemented with oncostatin M (10 ng/mL) according to the manufacturer's protocol. Other iPS cells were subjected to the three-step protocol [15, 17, 18]. They were cultured in RPMI 1640 medium supplemented with B27 and activin A (100 ng/mL) for 5 days to induce endodermal cell formation. The endodermal cells formed were then treated with BMP2 (30 ng/mL) and FGF4 (20 ng/mL) for 5 days, followed by HGF (20 ng/mL) for an additional 5 days. To induce hepatic maturation, cells were cultured for 5 days in HCM containing oncostatin M (10 ng/mL), as in our new protocol. In both protocols, cells were maintained in HCM with no oncostatin M after hepatic cell maturation. The growth medium was replaced every other day.

Immunocytochemical staining

Cells were fixed in 2% paraformaldehyde for 30 min and then permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) for 10 min. They were next incubated with primary antibody for 1 h at room temperature. Alexia Fluor 488- and 594-conjugated secondary antibodies (Invitrogen) were applied to the cells for 1 h at room temperature. Cell nuclei were stained with 4′,6-diamino-2-phenylindole (DAPI)

(Vector Laboratories, Burlingame, CA) and the cells were mounted using cover slips.

RNA isolation, reverse transcription, and quantitative real-time (RT)-PCR

Total cellular RNA was isolated using the Trizol Reagent (Invitrogen) and was used as a template for cDNA synthesis, performed using the superscript III first-strand synthesis system (Invitrogen). Quantitative real-time PCR was performed using the TaqMan gene expression system and SYBR Green (Applied Biosystems, Foster City, CA). Target gene expression values were normalized to those for the housekeeping gene GAPDH, and relative expression levels calculated by the $\Delta\Delta C_T$ method ($\Delta\Delta C_T = \Delta C_{Tsample} - \Delta C_{Tgapdh}$). The following primers were used: albumin, (forward) 5′-GCC TGC TGA CTT GCC TTC ATT AG-3′, (reverse) 5′-TCA GCA GCA GCA CGA CAG AGT A-3′; HNF4α, (forward) 5′-GGA ACA TAT GGG AAC CAA CA-3′, (reverse) 5′-AAC TTC CTG CTT GGT GAT GG-3′; CYP7A1, (forward) 5′-TGT TCA GGA CTG CGC ACA ATG-3′, (reverse) 5′-AGG ATT GCC TTC CAA GCT GAC-3′; CYP3A4, (forward) 5′-CAT TCC TCA TCC CAA TTC TTG AAG T-3′, (reverse) 5′-CCA CTC GGT GCT TTT GTG TAT CT-3′; and GAPDH, (forward) 5′-ATC AAC GAC CCC TTC ATT GAC C-3′, (reverse) 5′-CCA GTA GAC TCC ACG ACA TAC TCA GC-3′.

Urea assay

Cell culture supernatant urea concentrations were screened using a colorimetric assay (Bio Vision, Mountain View, CA), according to the manufacturer's instructions. Absorbances were measured at 570 nm using a microplate reader (model 680; Bio-Rad, Hercules, CA).

Enzyme-linked immunosorbent assay (ELISA) for albumin secretion

Levels of human albumin in cell culture supernatants were determined using a human albumin ELISA quantitation set (Bethyl Laboratory, Montgomery, TX) according to the manufacturer's instructions.

Cytochrome P450 (CYP) activity assay

CYP3A4 activity was assessed using a non-lytic method involving the P450-GLO screening system (Promega, Madison, WI) according to manufacturer's instructions. Cells were incubated with luminogenic substrate at 37°C for 1 h. CYP activity was then determined using a luminometer (Lumat LB9507; EG&G Berthold, Bad Wildbad, Germany) as relative light units per mL of tissue culture medium (RLU/mL).

Periodic acid Schiff (PAS) staining for glycogen

To detect glycogen, differentiated cells were stained using a PAS staining kit (Muto Pure Chemicals, Tokyo, Japan). Briefly, cells were fixed in 4% paraformaldehyde for 10 min, and were then oxidized through incubation with 1% periodic acid solution for 10 min. They were then incubated with freshly prepared Schiff's reagent at 37°C for 30 min. After the cells were rinsed with sulfurous acid, nuclei were counterstained through incubation with Mayer's hematoxylin for 5 min.

Results

New two-step protocol to drive iPS cells to differentiate into hepatic cells

A protocol for inducing iPS cells to differentiate into hepatic cells was recently described [15, 17]. It comprises three steps: (1) the treatment of iPS cells with 100 ng/mL activin A for 5 days to induce their differentiation into endodermal cells; (2) the treatment of iPS-derived endodermal cells with 30 ng/mL FGF4 and 20 ng/mL BMP2 for 5 days; and (3) incubation of the cells with 20 ng/mL HGF for 5 days to generate hepatic cells (Fig. 1a). When hepatic maturation was required, the cells were cultured in HCM supplemented with 10 ng/mL oncostatin M for a further 5 days (Fig. 1a). Although this protocol is relatively simple, it uses several cytokines, which are expensive and may produce unwanted effects, and is time-consuming—at least 2 weeks are required to generate hepatic cells.

To develop a method to generate hepatic cells from iPS cells using fewer cytokines, we referred to several protocols describing the generation of hepatocytes from embryonic stem (ES) cells [18–22]. Of the factors tested, the combination of activin A and HGF was found to be most effective in inducing the differentiation of human ES cells into hepatic cells [21]. Thus, we adapted this previous method to the generation of hepatic cells from human iPS cells. Following treatment with 100 ng/mL activin A for 3 days, cells were cultured with 20 ng/mL HGF for 5 days to generate hepatic cells. Thus, our protocol for generating hepatic cells from human iPS cells uses only two factors: high-dose activin A and HGF (Fig. 1b, c).

Efficient differentiation of iPS cells into ectoderm cells in the new two-step protocol

In our study, we used a high dose of activin A (100 ng/mL), known to efficiently induce definitive endoderm formation

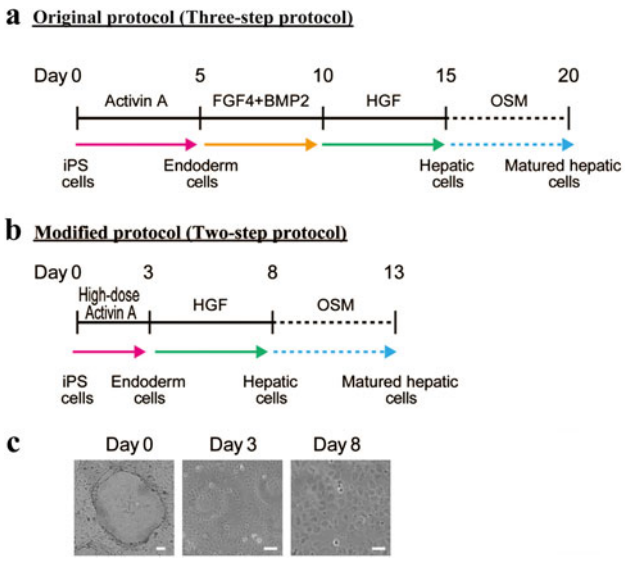

Fig. 1 Original and new protocols for the differentiation of iPS cells into mature hepatic cells. **a** Schematic representation of the original three-step protocol. iPS cells were cultured in RPMI 1640 medium supplemented with B27 and activin A (100 ng/mL) for 5 days to induce endoderm formation. Next, the cells were treated with FGF4 (30 ng/mL) and BMP2 (20 ng/mL) for 5 days. Subsequently, HGF (20 ng/mL) was added to the culture medium for 5 days to generate hepatic cells. To induce maturation, the hepatic cells were cultured in HCM containing oncostatin M (OSM; 10 ng/mL) for a further 5 days; **b** schematic representation of the modified two-step protocol used in the present study. iPS cells were cultured in RPMI 1640 medium supplemented with B27 and activin A (100 ng/mL) for 3 days to induce endoderm formation. Subsequently, cells were treated with HGF (20 ng/mL) for 5 days to generate hepatic cells. To achieve hepatic maturation, the hepatic cells were cultured in HCM containing OSM (10 ng/mL) for 5 days (as in the three-step protocol). The activin A incubation period was shorter, and culture with FGF4 and BMP2 was unnecessary, in this modified two-step protocol; **c** phase contrast microscopy demonstrating the typical iPS colony morphology (day 0), iPS cell-derived endoderm cell morphology (day 3) and iPS cell-derived hepatic-like cell morphology (day 8) in the two-step differentiation protocol. *Scale bar* 500 μm

[23, 24]. In fact, after treatment with activin A for 3 days, the iPS cells began to express FOXA2—a well-established endodermal marker—that displayed nuclear, but not nucleolar, immunolocalization (Fig. 2a). In addition, Sox17, another established endoderm marker, was detected (Fig. 2a), confirming that the cells were endodermal. The staining of these endodermal markers was detected in 50–75% of cells formed from two different parental iPS cell lines (Fig. 2b), suggesting that the observed phenomena were not cell type-specific. These results indicate that a high dose of activin A was sufficient to induce the differentiation of iPS cells into endodermal cells, consistent with reports of similar outcomes using ES cells [23, 24].

Levels of AFP and albumin expression in the hepatic cells generated using the new protocol were comparable to those in cells produced using the three-step protocol

After treatment with HGF for 5 days, we evaluated the cells' differentiation status by immunostaining for the hepatic cell markers AFP and albumin. As shown in Fig. 3, both AFP and albumin were expressed in the cytoplasm in about 40% of cells (Fig. 3a, b). Numbers of AFP- and albumin-expressing cells were comparable when our new two-step protocol and the original three-step protocol were employed (Fig. 3b), and were similar to those reported previously [15]. These results suggest that the endodermal cells generated using our two-step protocol differentiated into hepatic cells with similar efficiency to those generated using the original three-step protocol.

Hepatic function of the induced hepatic cells generated using the new protocol was comparable to that of cells produced using the three-step protocol

Although cells treated with activin A and HGF expressed hepatic markers, such as AFP and albumin, levels of albumin secreted into the culture medium were too low to be detected by ELISA (data not shown). To induce additional hepatic maturation, cells were cultured in HCM supplemented with 10 ng/mL oncostatin M for further 5 days [25, 26]. To compare the hepatic cells' differentiation status, urea production (Fig. 4a, left panel), albumin secretion (Fig. 4a, right panel), CYP3A4 activity (Fig. 4b), and glycogen deposition (Fig. 4c) were examined in the cells generated from two different parental iPS cells using the three-step protocol and our new two-step protocol. Cells generated using the two protocols were found to display comparable hepatic function (Fig. 4a, c). In fact, our new protocol may be slightly superior in terms of hepatic function of the cells generated. Consistent with these findings, mRNA expression of a panel of hepatic markers, consisting of albumin, HNF4α, CYP3A4, and CYP7A1, was also comparable between cells generated using the three-step protocol and our new protocols (Fig. 4d). In particular, CYP7A1 expression is used to indicate that those cells are derived from definitive endoderm cells [27]. These results suggest that our protocol, using only two factors (activin A and HGF) is as efficient at generating hepatic cells from iPS cells as the three-step protocol using four factors (activin A, FGF4, BMP, and HGF). In addition, omitting the FGF4 and BMP2 treatment step shortens the protocol's duration.

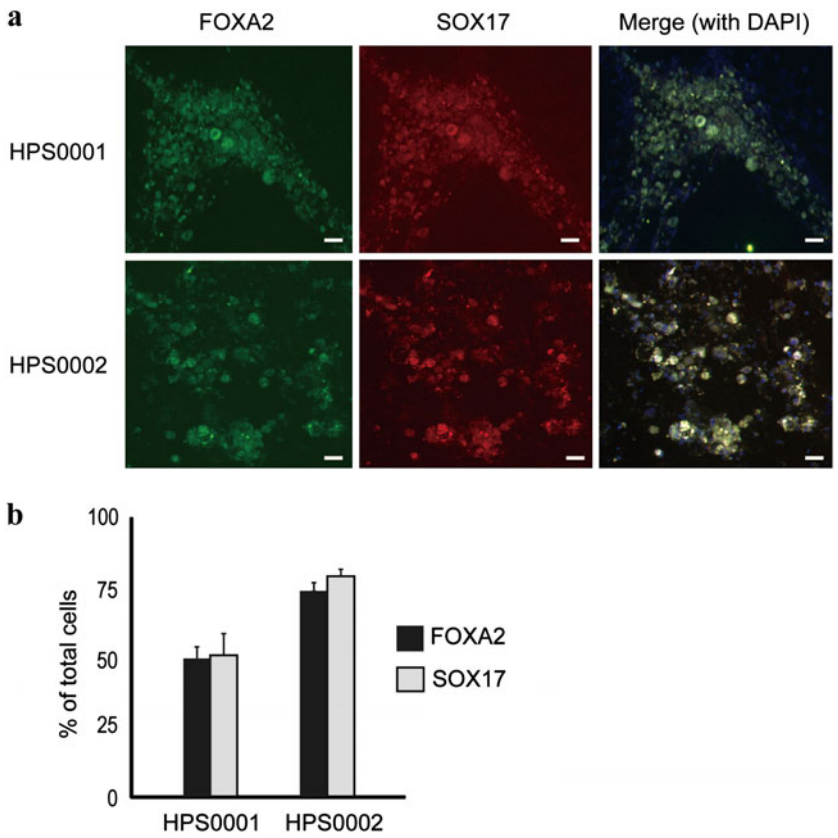

Fig. 2 Endodermal marker staining after the generation of endoderm from iPS cells using our modified protocol. **a** Immunocytochemical staining for endodermal markers after induction of endoderm formation using our two-step protocol. iPS cells were treated with activin A (100 ng/mL) for 3 days to induce their differentiation into endoderm. Differentiated cells were stained with antibodies raised against the endodermal markers FOXA2 (*green*) and SOX17 (*red*). Nuclei were counterstained with DAPI. Two human iPS cell lines, HPS0001 (*upper panels*) and HPS0002 (*lower panels*), were used. Results shown are representative of two independent experiments. *Scale bar* 500 μm; **b** numbers of cells expressing endodermal markers at day 3 after the start of induction. Data show the proportions of DAPI-positive cells that were FOXA2-positive or SOX17-positive. No obvious difference in the proportions was detected between the two parental iPS cell lines analyzed (HPS0001 and HPS0002). Data represent the mean ± SD of two independent experiments

Discussion

We report here that human iPS cells can be induced to differentiate into hepatic cells through sequential treatment with only two factors, activin A and HGF. This method is as efficient as previously described protocols. In addition, it requires a shorter culture period.

Our protocol used a high dose of activin A to induce the formation of definitive endoderm from iPS cells, similar to a previous protocol using ES cells [23, 24]. Nodal, a member of the TGF-β super family, directs the formation of definitive endoderm [28]. Previous studies reported that activin A binds to the same receptor as Nodal and that high doses of activin A effectively induce the formation of definitive endoderm from human ES cells [23, 24]. These results were the rationale for our use of a high dose of activin A to generate endodermal cells from iPS cells. In fact, we were similarly able to induce iPS cells to differentiate into endodermal cells using only a high dose of activin A.

The second cytokine used in this study, HGF, which is critical for the development and regeneration of the liver, appears to be essential for driving endodermal cells to differentiate into hepatic cells [29–31]. We used HGF to differentiate our induced endodermal cells into hepatic cells. The hepatic cells generated using our protocol exhibited differentiation comparable to those produced using the three-step protocol, as assessed by expression of the hepatic cell markers AFP and albumin. This suggests that HGF is sufficient to induce the differentiation of endodermal cells into hepatic cells. A recent study showed that hepatocyte-like cells could be generated from iPS cells using FGF4 and BMP2 to induce endodermal cells to differentiate along the hepatic lineage [15]. We found these cytokines to be non-essential. Instead, our results are consistent with those of another recent study that focussed on the generation of hepatic cells from human ES cells [21]. In that study, activin A plus HGF was found to be the most effective combination for inducing the differentiation

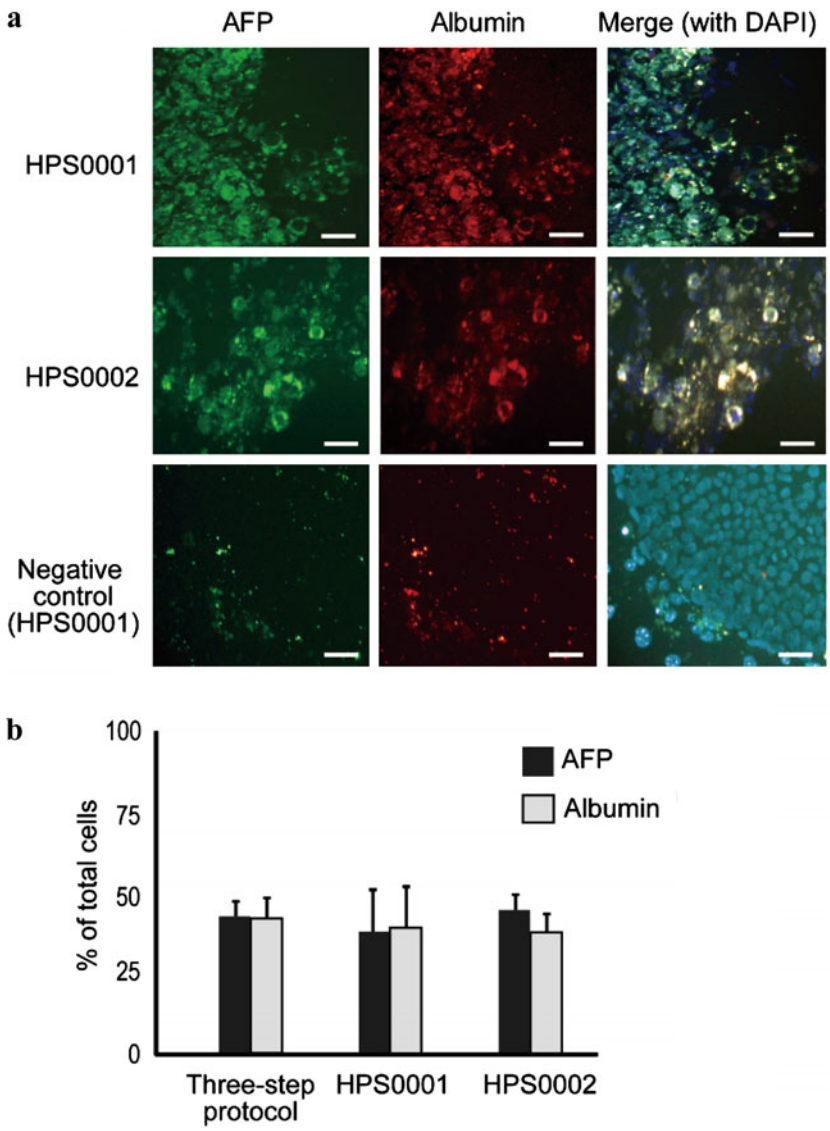

Fig. 3 Hepatic marker-staining in the hepatic cells generated using our two-step protocol. **a** Immunocytochemical staining using antibodies specific for hepatic cell markers. Human iPS cell-derived hepatic cells generated using our two-step protocol were stained at day 8 with anti-AFP (*green*) and anti-albumin (*red*) antibodies. Non-induced iPS cells (HPS0001) were stained similarly as a negative control. Nuclei were counterstained with DAPI. Results shown are representative of two independent experiments. *Scale bar* 500 μm; **b** differences in parental iPS cells do not influence the proportion of cells expressing hepatic cell markers after differentiation. Data show the proportions of DAPI-positive cells that were AFP-positive or albumin-positive 8 days after the induction of hepatic cell differentiation. No obvious difference in the proportions was detected between cells derived from the HPS0001 and HPS0002 iPS cell lines. Data represent the mean ± SD of three independent experiments

of human ES cells into AFP-positive and albumin-positive hepatic cells (combinations of other cytokines including activin A, FGF, BMP, and HGF were also tested). Moreover, FGF/BMP reduced the effects of activin A and HGF on hepatic differentiation [21]. In vertebrate embryos, hepatogenesis from the endoderm layer has been reported to begin on embryonic day 8, triggered by FGFs produced by the cardiac mesoderm [32]. However, a previous study demonstrated that FGFs have much smaller effects on the transition from albumin-negative stem cells to albumin-positive cells in fetal hepatic cell differentiation [25]. Although FGFs may directly induce the differentiation of hepatic cells from foregut endoderm, HGF also stimulates the differentiation of dormant stem cells. Moreover, HGF has strong stimulatory effects on the differentiation of hepatic cells from hematopoietic stem cells [33] and hES cells [34]. Thus, HGF may play important roles in hepatic cell differentiation and proliferation. In addition, the results of previous studies suggested that HGF directly regulates the expression of C/EBPα, which regulates liver-specific gene expression and cell proliferation and also promotes the differentiation of stem cells into hepatic cells [35]. In fact, previous studies demonstrated efficient maturation of hepatic cells generated from human ES cells using only HGF [21], and of hepatic cells generated from mouse fetal hepatic cells using a combination of HGF with oncostatin M [25]. These observations may support our use of activin A and HGF, without FGF4 or BMP2, to efficiently generate hepatic cells from iPS cells.

Although the methods used to generate hepatic cells from iPS cells are continually being improved, further studies are needed to optimize the induction and culture protocols used to produce, from induced hepatic cells, mature hepatocytes that may be used in regenerative

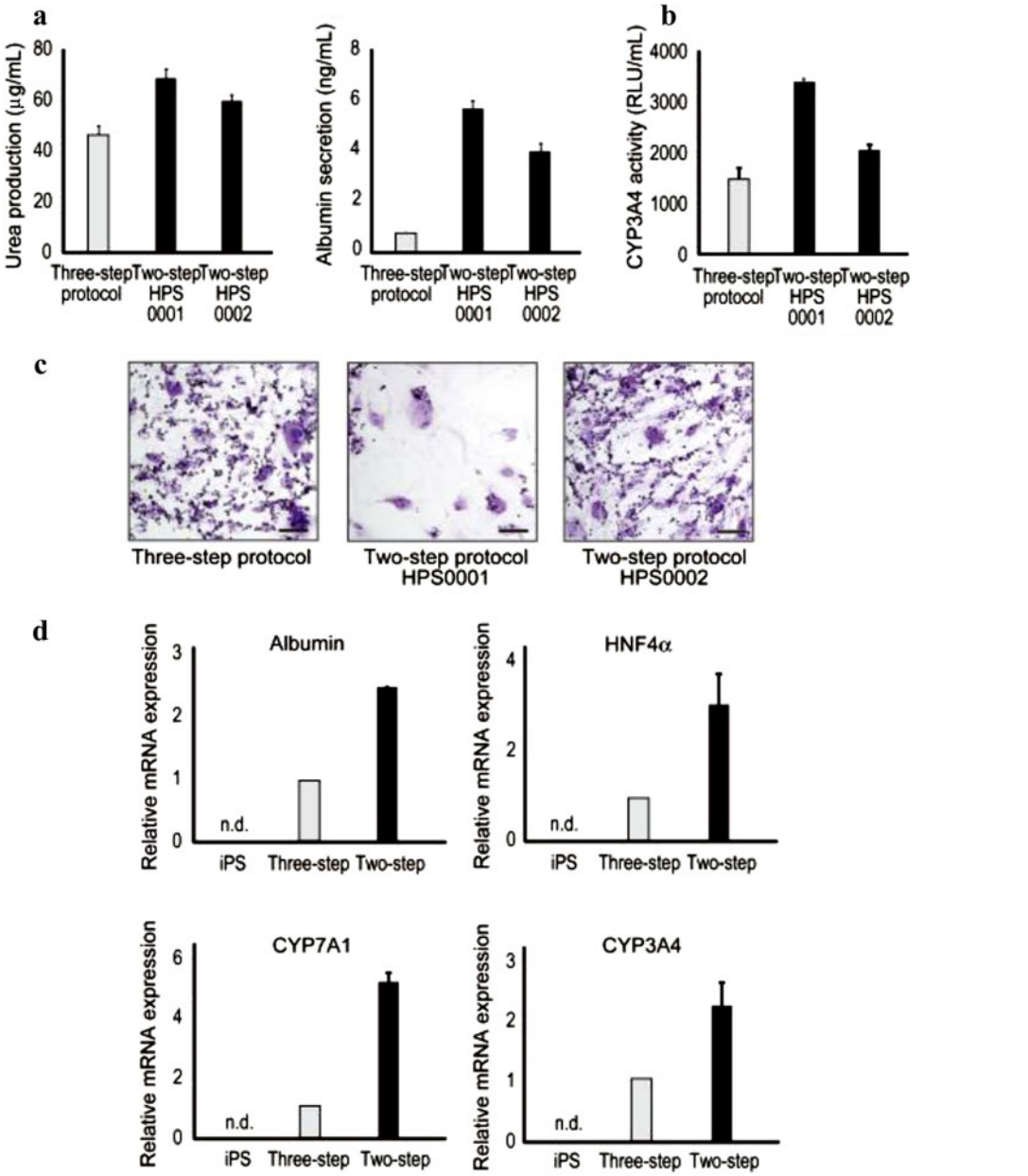

Fig. 4 Comparison of hepatic function in hepatic cells generated using our two-step protocol and the original three-step protocol. **a** Urea production and albumin secretion by human iPS cell-derived hepatocytes. At day 15 after the start of induction, levels of urea (*left panel*) and albumin (*right panel*) were measured in cell culture supernatants obtained after 48 h of cell growth. Similar amounts of urea and greater amounts of albumin were released from hepatic cells generated from HPS0001 and HPS0002 cells using our two-step protocol compared with those released from hepatic cells generated from HPS0001 cells using the original three-step protocol. Data represent the mean ± SD of three independent experiments; **b** cytochrome P450 activity in human iPS cell-derived hepatic cells. CYP3A4 activity was assessed by measuring the enzymatic release of free luciferin from an inactive luciferin precursor. Similar or slightly higher CYP3A4 activity was observed in hepatic cells produced using our two-step protocol compared with that in cells generated using the original three-step protocol. The results are expressed as relative light units per mL of tissue culture medium (RLU/mL); **c** glycogen synthesis in human iPS cell-derived hepatic cells. Glycogen stored in the cytoplasm is stained red-purple by PAS. Staining of stored glycogen was comparable in hepatic cells generated from HPS0001 and HPS0002 cells using our two-step protocol and from HPS0001 cells using the original three-step protocol. Nuclei were counterstained with hematoxylin. Results shown are representative of two independent experiments. *Scale bar* 500 μm; **d** quantitative RT-PCR analysis of albumin, HNF4α, CYP7A1, and CYP3A4 mRNA expression in non-differentiated iPS (HPS0001) cells and hepatic cells generated from iPS cells using the original three-step protocol and our two-step protocol. Values were normalized to the levels of GAPDH mRNA (internal control). Values (mean ± SD of triplicate data) represent relative expression ratios, with the values for hepatic cells generated using the three-step protocol being set to 1 (*nd* not detected)

cell-based therapies. Nonetheless, the protocol we have described here provides a simple, cost-effective, and time-saving approach for generating hepatic cells from iPS cells. We hope that it will facilitate the future use of iPS cells in the field of hepatology.

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