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ORIGINAL ARTICLE

TERM

Functional Comparison of Human Embryonic Stem Cells and Induced Pluripotent Stem Cells as Sources of Hepatocyte-Like Cells

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Pluripotent stem cells can differentiate into many cell types including mature hepatocytes, and can be used in the development of new drugs, treatment of diseases, and in basic research. In this study, we established a protocol leading to efficient hepatic differentiation, and compared the capacity to differentiate into the hepatocyte lineage of human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs). Optimal combinations of cytokines and growth factors were added to embryoid bodies produced by both types of cell. Differentiation of the cells was assessed with optical and electron microscopes, and hepatic-specific transcripts and proteins were detected by quantitative reverse transcription polymerase chain reaction and immunocytochemistry, respectively. Both types of embryoid body produced polygonal hepatocyte-like cells accompanied by time-dependent up regulation of genes for α-fetoprotein, albumin (ALB), asialoglycoprotein1, CK8, CK18, CK19, CYP1A2, and CYP3A4, which are expressed in fetal and adult hepatocytes. Both types of cell displayed functions characteristic of mature hepatocytes such as accumulation of glycogen, secretion of ALB, and uptake of indocyanine green. And these cells are transplanted into mouse model. Our findings indicate that hESCs and hiPSCs have similar abilities to differentiate into hepatocyte *in vitro* using the protocol developed here, and these cells are transplantable into damaged liver.

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Key Words: Hepatocyte differentiation; Hepatocyte-like cells; Human embryonic stem cells; Human induced pluripotent stem cells

INTRODUCTION

Recently Yamanaka and others have generated induced pluripotent stem cells (iPSCs) from human and mouse fibroblasts by incubating the fibroblasts with four defined factors [1,2]. The use of embryonic stem cells (ESCs) and iPSCs has received attention as cell replacement therapy owing to their ability to differentiate into hepatocytes *in vitro* [3-7]. For practical application of ESCs and iPSCs, straightforward and reliable *in vitro* [8-11] and *in vivo* [12] differentiation protocols are needed. *In vitro* research using embryoid bodies has focused on creating the right microenvironment for hepatogenesis [13,14] by adding agents and cytokines that promote the formation of hepatocytes, such as fibroblast growth factor (FGF), hepatocyte growth factor, oncostatin M, insulin-transferrin-selenium, retinoic acid, and β-nerve growth factor [3,15]. Also, introduction of genes promoting endodermal differentiation [16], extracellular matrix proteins [17], and co-culture with other cell types [18] are being used to induce differentiation of ESCs and iPSCs into the hepatocyte lineage. Nonetheless, the established methods are generally incapable of providing enough hepatocyte precursors for clinical use.

The stem cell properties, self-renewal capacity and pluripotency of ESCs and iPSCs are very similar, but whether there are some differences in gene expression profiles is unclear. Small groups of genes appear to be differentially expressed and this might affect the functional properties of the ESCs and iPSCs [19-21].

In this paper, we investigate whether iPSCs and ESCs have the same potential to differentiate into hepatocyte-like cells (HLCs).

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MATERIALS AND METHODS

Cell culture

Undifferentiated hESCs (UCO6) and iPSCs (iPSW) were cultivated on mouse embryonic fibroblast feeder layers in DMEM/F12 medium supplemented with 20% KnockOut serum replacement (Gibco, Carlsbad, CA, USA), 1 mM nonessential amino acids (Gibco), L-glutamine (Gibco), penicillin/streptomycin (Gibco), 0.55 mM 2-mercaptoethanol (Sigma, St. Louis, MO, USA) and 4 ng/mL recombinant human FGF2 (R&D systems, Minneapolis, MN, USA). Cells were passaged manually at 1:3−1:10 dilutions every 5−7 days. Initial differentiation was in RPMI (Gibco) supplemented with glutamine and 0.5% FBS (Hyclone, Logan, UT, USA) after allowing embryoid body (EB) formation for one day.

RNA Isolation and RT-PCR Analysis

Total RNA was obtained with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Polymerase chain reaction (PCR) products were separated on 1.2% agarose gels containing ethidium bromide. The primer sequences and optimal annealing temperatures used are shown in Supplementary Table 1 (in the online-only Data Supplement). Quantitative real-time PCR was conducted with a SYBR Green PCR Core Reagent kit (Applied Biosystem, Foster City, CA, USA) [22].

Immunocytochemistry

Cultured cells were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) at room temperature for 20 min, washed in PBS, and permeabilized with buffer containing 0.3% Triton X-100 and 3 % BSA in PBS for 1 hour at room temperature. Cells were incubated with first antibody at 4°C overnight, and then treated with fluorochrome-conjugated secondary an-

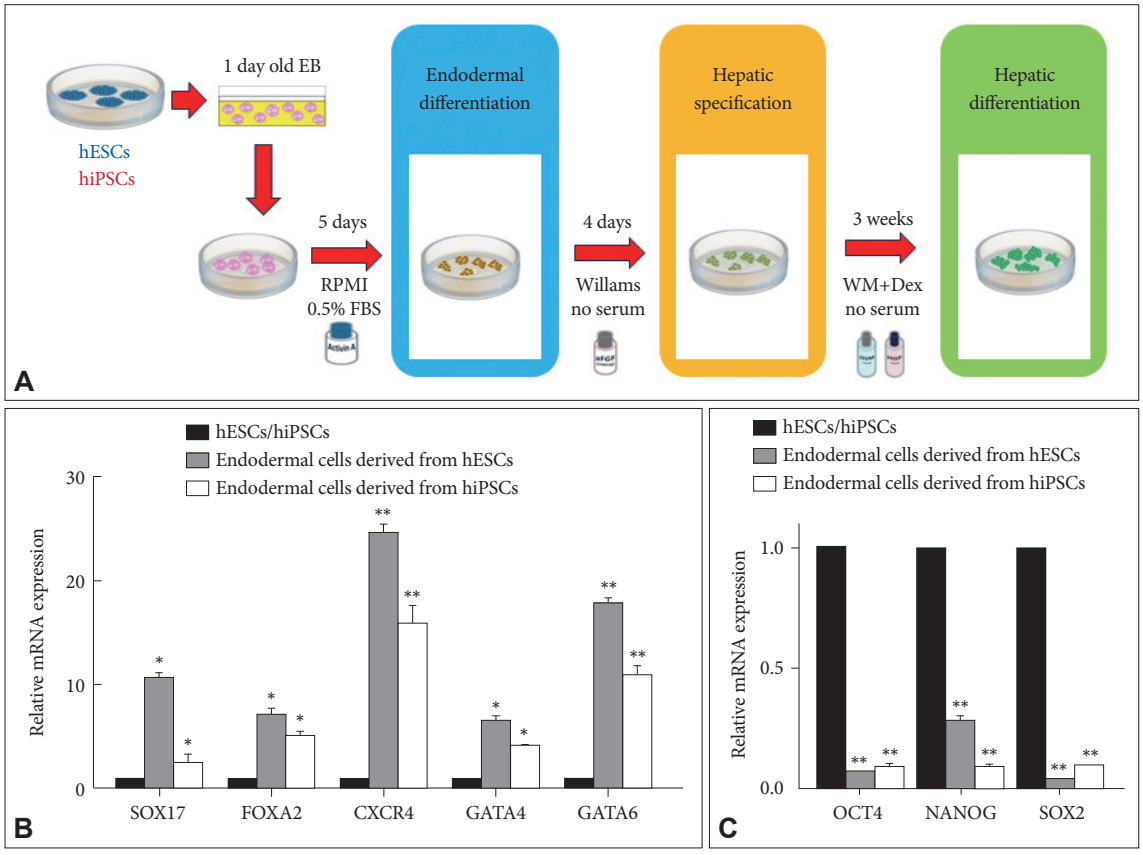

Figure 1. Production of definitive endoderm lineage cells from hESCs and hiPSCs. (A) Schematic procedure for differentiation of hiPSCs and hESCs into hepatocyte-like cells. One-day EBs from hESCs and hiPSCs were incubated with several growth factors and cytokines and formed hepatocyte-like cells. (B) Detection of endoderm-specific gene expression in the endodermal cells derived from hESCs (gray bars) and hiPSCs (white bars). Black bar refers to pluripotent stem cells as a control. The results are expressed as the means±SDs of three independent experiments. *p<0.05, **p<0.01 compared with the pluripotent stem cell control (black bars). (C) Detection of pluripotent-cell-specific gene expression in endodermal cells derived from hESCs (gray bars) and hiPSCs (white bars). Black bar refers to pluripotent stem cells as control. The results are expressed as the means±SD of three independent experiments. **p<0.01 compared with the pluripotent stem cell control (black bars). hESCs: human embryonic stem cells, hiPSCs: human induced pluripotent stem cells, EB: embryoid body, SD: standard deviations. (Continued to the next page)

tibody (1:250; Jackson Immunoresearch Labs, West Grove, PA, USA). The primer sequences and optimal annealing temperatures used are given in Supplementary Table 2 (in the online-only Data Supplement). Antibody labeling was conducted at room temperature for 2 hours. Cells on culture dishes were mounted using mounting medium with DAPI (Vector Labs, Burlingame, CA, USA) and examined with fluorescence and confocal laser scanning microscopes (Carl Zeiss, Oberkochen, Germany).

Electron microscopy

Thin sections of cultured cells were made by the *in situ* method for EM analysis as previously described [3]. Briefly, cells on 6-well plates were fixed by overlaying 2% glutaraldehyde in sodium cacodylate buffer (0.1 M, pH 7.0), rinsed in the same buffer, and post-fixed with 1% osmium tetroxide. The cells were then rinsed in cacodylate buffer, followed by acetate buffer (0.1 M, pH 4.0), and *en bloc* stained in 0.5% uranyl acetate in acetate buffer for 1 hr. They were again rinsed in acetate buffer followed by cacodylate buffer, and dehydrated in an ethanol series. After three changes of 100% ethanol, the cells were washed with three changes in pure epoxy resin, infiltrated overnight, embedded in epoxy resin, and cured in a 55°C oven for 48 hours. The cured epoxy moulds were separated from the plates after submerging in liquid nitrogen. Thin sections at 70 to 80 nm were cut parallel to the direction of growth of the cells using a diamond knife and ultra-microtome. The sections were mounted on 200 mesh grids and stained in uranyl acetate and lead citrate. They were examined and imaged with a TEM equipped with a CCD camera (JEM-2000EX, JEOL USA, Inc., Peabody, MA, USA).

Evaluation of metabolic activity and hepatic protein secretion

We measured secretion of albumin (Bethyl Laboratories, Montgomery, TX, USA), and urea (Abcam, Cambridge, UK)

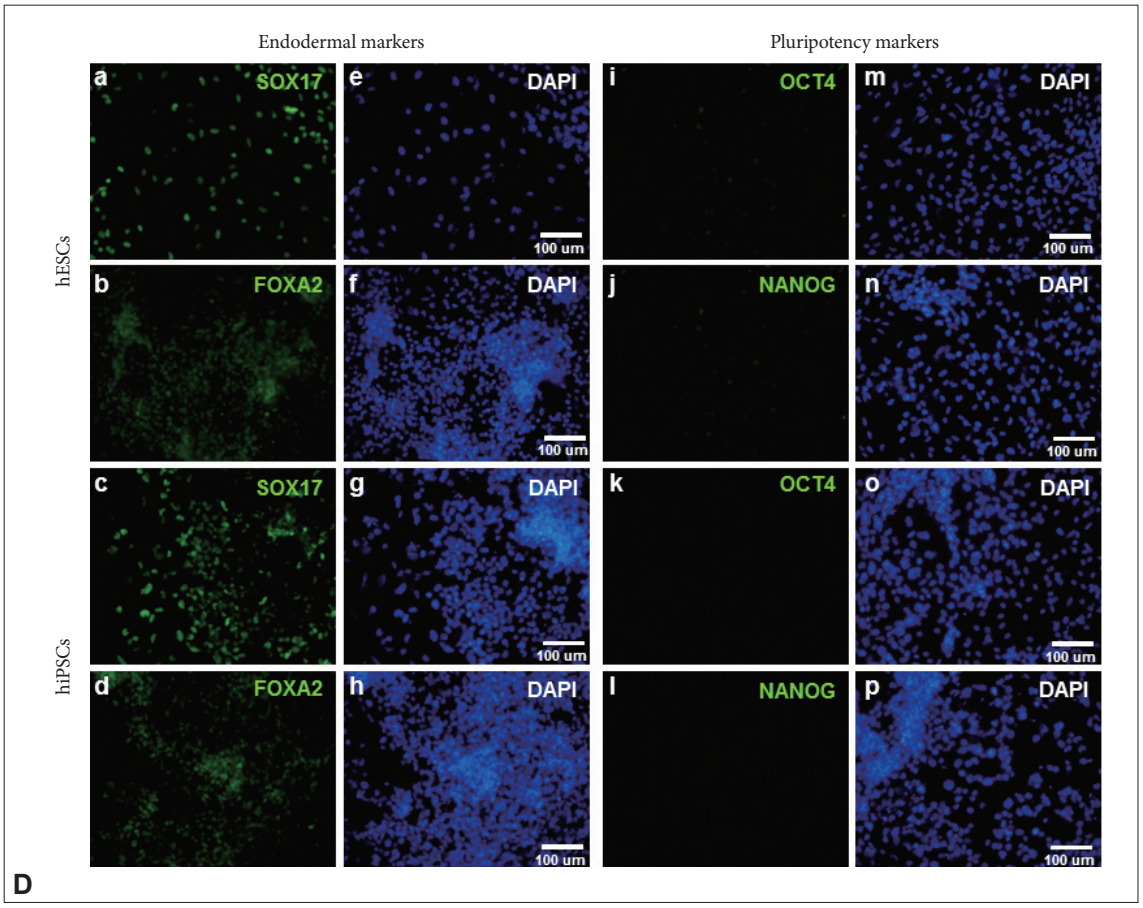

Figure 1. (Continued from the previous page) Production of definitive endoderm lineage cells from hESCs and hiPSCs. (D) Immunofluorescence analysis of differentiated definitive endoderm cells. (a-d) Immunostaining of endoderm marker proteins (SOX17, FOXA2) of hESCs and hiPSCs; (i-l) immunostaining of pluripotent cell marker proteins (OCT4, NANOG) in hESCs and hiPSCs. DAPI (e-h, m-p) labels all nuclei. Scale bar, 100 um. hESCs: human embryonic stem cells, hiPSCs: human induced pluripotent stem cells, DAPI: 4',6-diamidino-2-phenylindole.

into the culture medium over 24 hour using a human albumin enzyme-linked immunosorbent assay (ELISA) kit. A periodic acid Schiff (PAS) staining kit (Sigma-Aldrich, St. Louis, MO, USA) was used to detect glycogen particles in the HLCs. For indocyanine green (ICG) uptake, 25 mg of ICG (Sigma-Aldrich) was dissolved in 5 mL of solvent in a sterile vial and added to 20 mL of medium to a final concentration of 1 mg/mL. The ICG solution was added to the cells and incubated at 37°C for 30 minutes. After washing three times with PBS, the cellular uptake of ICG was examined under a microscope.

Transplantation

Homozygous major urinary protein-urokinase-type plasminogen activator/severe combined immunodeficiency (MUP-uPA/SCID) mice were generated by crossbreeding MUP-uPA transgenic mice. 10^6 hepatocyte like cells were injected into the inferior poles of the spleens of 3-week-old mice. One day after injection, liver was harvested for immunohistochemistry.

Immunohistochemistry

Liver tissues were fixed in 4% paraformaldehyde for 2 hours at 4°C, and the tissues were incubated in 30% sucrose overnight and embedded in OCT compound (Sakura, Osaka, Japan). Cryostat sections (8 um) were washed with PBS and permeabilized in PBS with 0.1% BSA, 0.3% Triton X-100, and 10% normal goat serum for 45 minutes at room temperature. Anti-human serum albumin (1:100; R&D system, Minneapolis, MN, USA), human AFP (DAKO, Santa Clara, CA, USA), and HNF4a (Santa Cruz Biotech, Dallas, TX, USA) were used for immunostaining. Slides were mounted with medium containing DAPI (Vector labs).

Statistical analysis

Quantitative data are presented as means±standard deviations, and as inferential statistics (*p* values). Statistical significance was evaluated using two-tailed t-tests with significance levels recorded as $*p<0.05$, $**p<0.01$, $***p<0.005$.

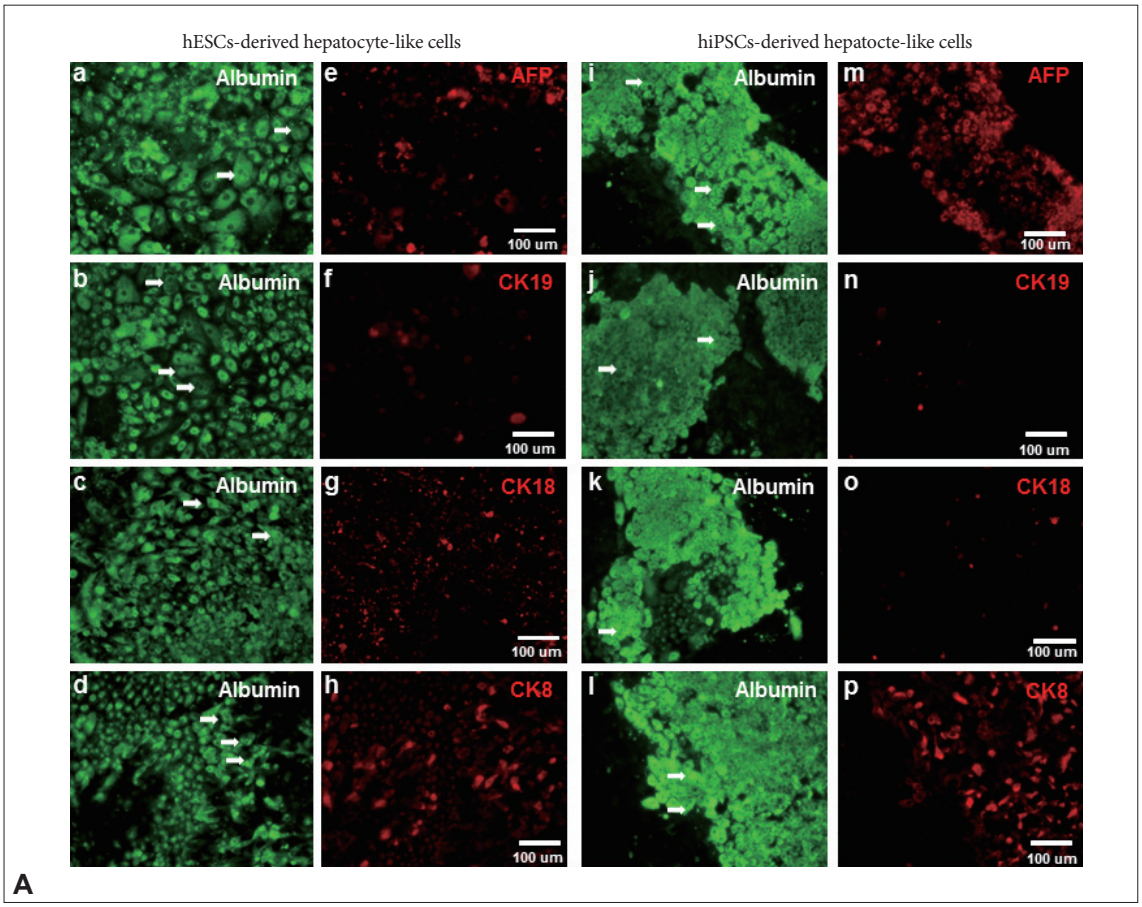

Figure 2. Characterization of HLCs derived from hESCs and hiPSCs. (A) Immunofluorescence analysis of HLCs using antibodies against albumin (a-d, i-l), AFP (e, m), CK19 (f, n), CK18 (g, o), and CK8 (h, p). Albumin-positive cells are co-stained with AFP, CK18, and CK8, not CK19. Scale bar, 100 um. hESCs: human embryonic stem cells, hiPSCs: human induced pluripotent stem cells, AFP: alpha-fetoprotein, HLCs: hepatocyte-like cells. (Continued to the next page)

RESULTS

In vitro differentiation of hESCs and hiPSCs into definitive endoderm lineage cells

In order to promote the differentiation of hESCs and hiPSCs into HLCs, we first induced them to differentiate into endoderm cells from embryoid bodies made up of pluripotent stem cells. One-day-old EBs derived from both types of cell were then plated on collagen-coated dishes and cultured for 5 days with activin A to form definitive endodermal cells (Fig. 1A). Endodermal marker genes such as SOX17, FOXA2, CXCR4, GATA4 and GATA6, were measured by real-time PCR (Fig. 1B), and expression of these markers was elevated in the endodermal cells derived from both types of cell, but it was higher in the hESC-derived endodermal cells. Also, expression of OCT4, Nanog and SOX2 decreased, indicating that the differentiated cells were definitive endodermal cells (Fig. 1C). To confirm the real-time PCR data, we stained the cells with antibodies to each protein (Fig. 1Da-p). The results confirmed the PCR data. Together, these results indicate that hESCs and hiPSCs can differentiate efficiently into definitive endodermal cells under optimal conditions, and these cells no longer express markers of pluripotent cells.

In vitro differentiation of hESCs and hiPSCs into hepatocyte-like cells

When the EB-derived endodermal cells were cultured in chemically-defined serum-free medium they generated distinct outgrowths; these expanded as monolayer cultures after treatment for 4 days with a cytokine cocktail designed to induce hepatic specification, and incubation without serum for 3 weeks to promote hepatic differentiation (Fig. 1A). Cells in these clusters expanded into sheets of tightly packed cells which displayed typical hepatocyte morphology, including binucleate cells (Fig. 2A), and they co-expressed albumin and AFP (Fig. 2Aa, e and i, m). These albumin-positive cells stained with CK18 and CK8 but not with CK19, indicating that they were hHLCs (Fig. 2A). Also, other hepatocyte-specific proteins, such

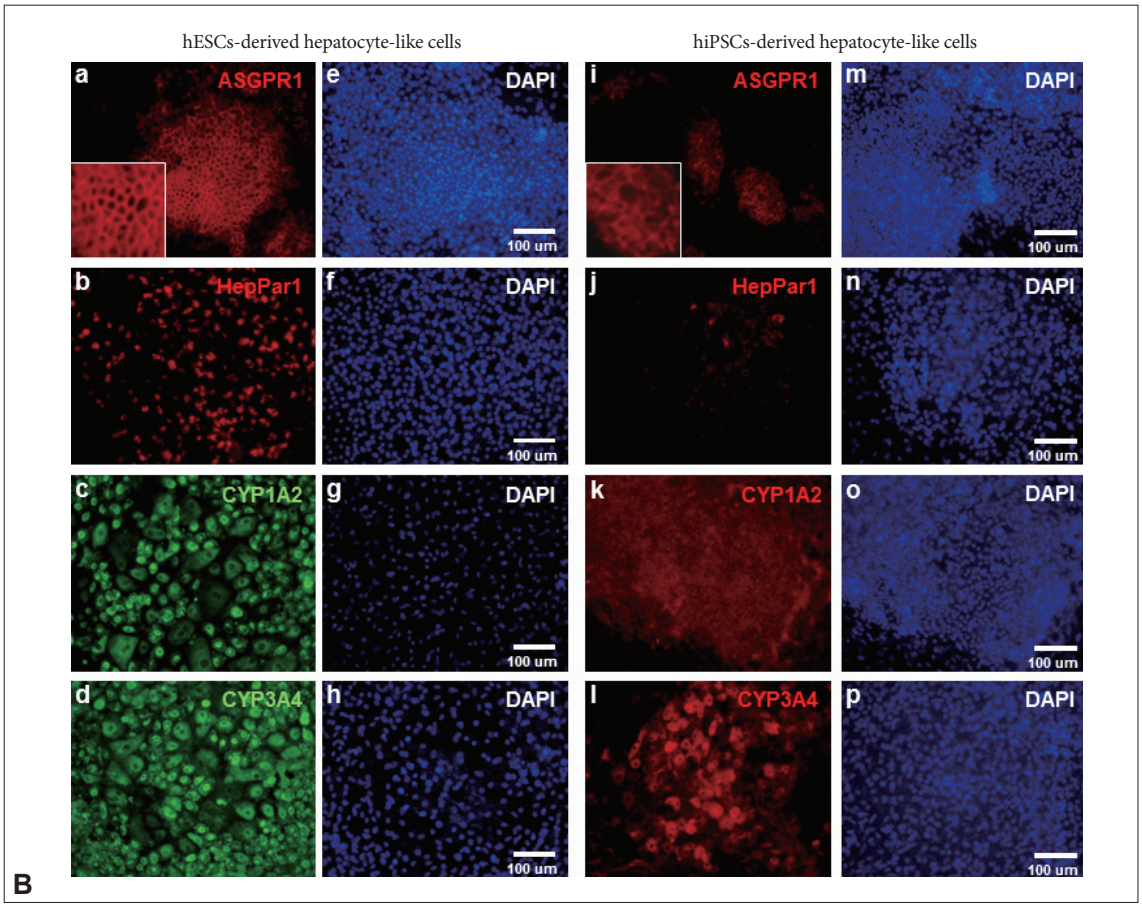

Figure 2. (Continued from the previous page) Characterization of HLCs derived from hESCs and hiPSCs. (B) Immunofluorescence analysis of HLCs using antibodies against ASGPR1 (a, i), HepPar1 (b, j), CYP1A2 (c, k), CYP3A4 (d, l). DAPI (e-h, m-p) labels all nuclei. Scale bar, 100 um. hESCs: human embryonic stem cells, hiPSCs: human induced pluripotent stem cells, HLCs: hepatocyte-like cells, DAPI: 4',6-diamidino-2-phenylindole.
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as ASGPR1, HepPar1, CYP1A2, CYP 3A4 were identified by staining (Fig. 2B) and real-time PCR showed that they expressed hepatocyte-specific genes (Fig. 2C). The clustered cells tended to have hepatocyte-like morphology and gave a typical PAS staining pattern (Fig. 3Aa, b and d, e). Cellular uptake of ICG is a unique characteristic of hepatocytes, and most of the hepatocytes formed by the human pluripotent stem cells took up ICG (Fig. 3Ac and f). These cells also secreted albumin into the culture medium (Fig. 3B).

Electron microscopy of hESC- and hiPS-derived hepatocytes

Electron microscopy showed that both hESC- and hiPSC-derived HLCs had typical hepatocyte features (Fig. 4). Polygonal cells with a high cytoplasm-to-nuclear ratios and large round euchromatic nuclei with prominent nucleoli were evident in the cultures (Fig. 4a and b; arrows indicate large round euchromatic nuclei). Numerous mitochondria and cytoplasmic vesicles, also characteristic of hepatocytes, were seen (Fig. 4a and b). Finally, the formation of bile canaliculi and abundant microvilli was evident in the cultures of both hESC- and hiP-

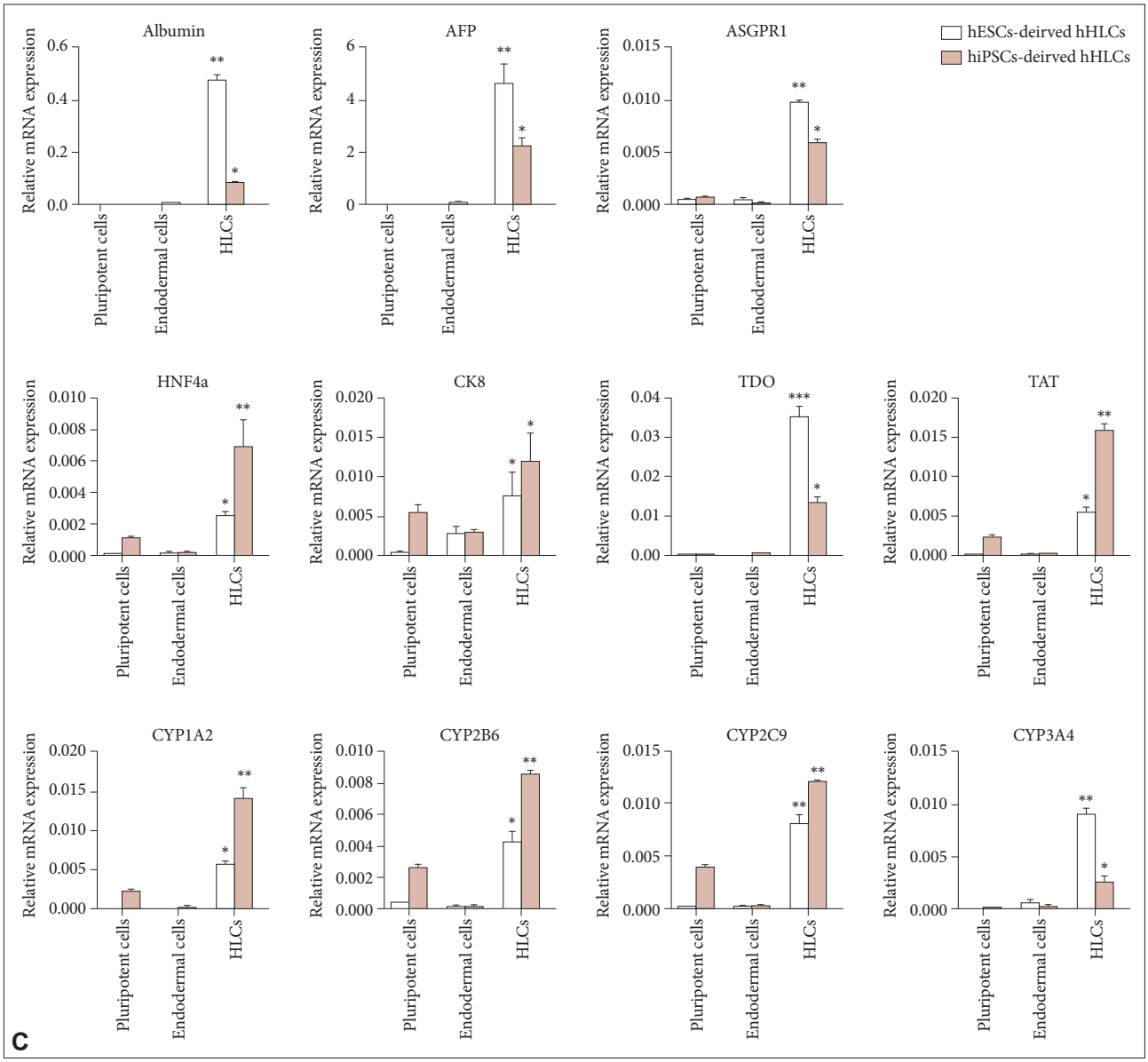

Figure 2. (Continued from the previous page) Characterization of HLCs derived from hESCs and hiPSCs. (C) Detection of hepatocyte-specific gene expression in HLCs derived from hESCs and hiPSCs. The results are expressed as the means±SD of three independent experiments. *p<0.05, **p<0.01, ***p<0.005 compared with pluripotent stem cells and endodermal cells. hESCs: human embryonic stem cells, hiPSCs: human induced pluripotent stem cells, HLCs: hepatocyte-like cells, SD: standard deviation.

SC-derived HLCs (Fig. 4c and f). These data suggest that the HLCs derived from hiPSCs and hESCs were morphologically and functionally equivalent to primary hepatocytes. It will be of interest to determine whether these hHLCs can substitute for normal hepatocytes for treating liver damage.

Transplantation of HLCs into a MUP-uPA/SCID Mouse

To evaluate *in vivo* transplantation function, GFP-expressing HLCs were injected into spleen of MUP-uPA/SCID mouse. One day post injection, harvested liver was stained with human albumin, AFP and HNF4A. In Figure 5, GFP expressing HLCs were founded (Fig. 5a, e, and i), and these cells were detected with human albumin (Fig. 5b), AFP (Fig. 5f) and HNF4A (Fig. 5j) antibodies. These results support that HLCs derived from pluripotent stem cells can be used liver regeneration as transplantation into spleen.

DISCUSSION

Pluripotent stem cells are a useful source of cells for organ therapy. Since the primary cells of viscus organs cannot be isolated, cells derived from pluripotent stem cells have been focused on as alternatives.

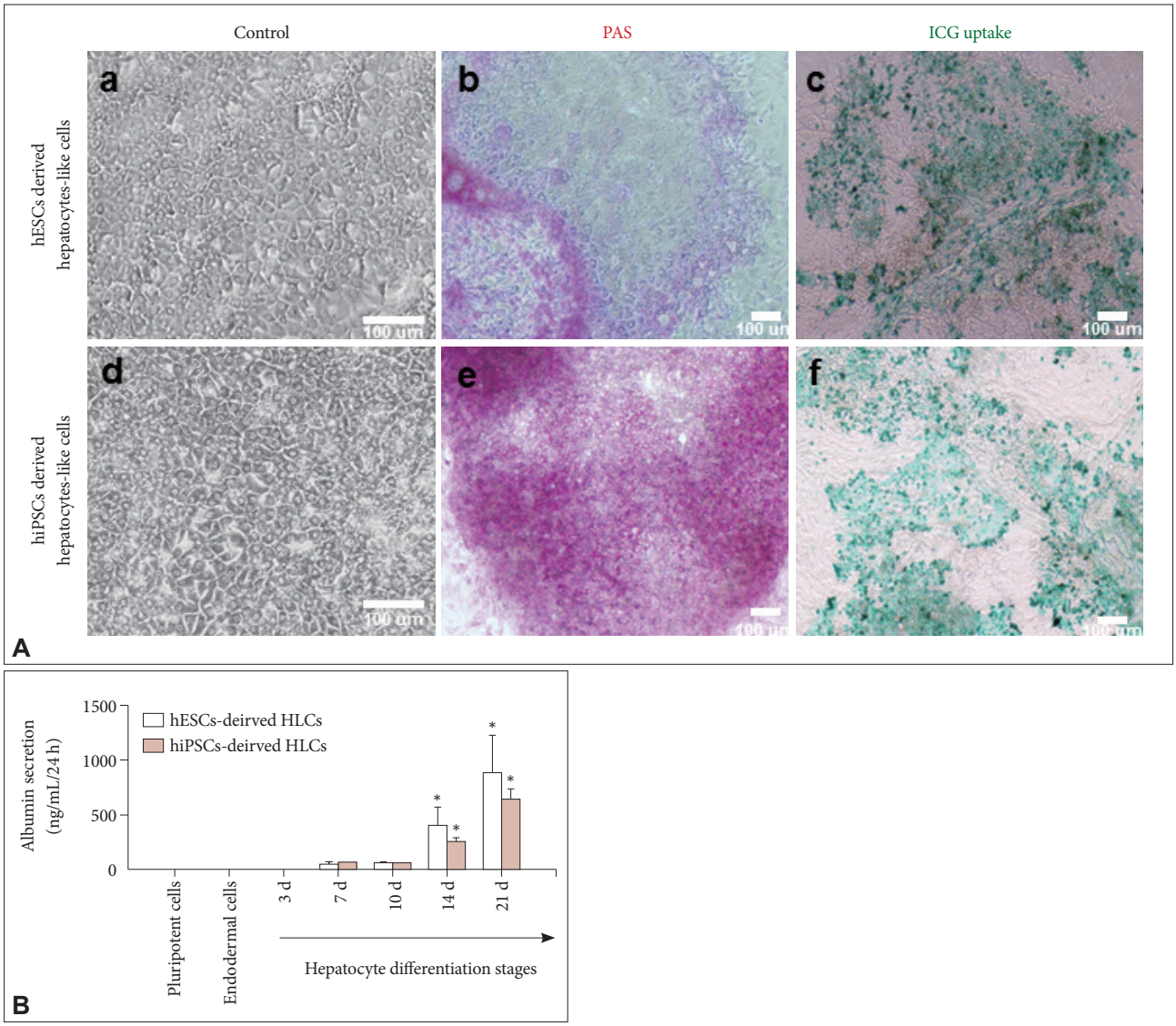

Figure 3. Functional analysis of HLCs. (A) Periodic acid-Shiff (PAS) staining of HLCs (b, e), and indocyanine green (ICG) uptake assays (c, f). The morphologies of HLCs derived from hESCs and hiPSCs are shown in a, d. Scale bar, 100 um. (B) Measurement of albumin secretion from HLCs derived from hESCs and hiPSCs. *p<0.05, compared with pluripotent stem cells and endodermal cells. hESCs: human embryonic stem cells, hiPSCs: human induced pluripotent stem cells, HLCs: hepatocyte-like cells.

Even though human hepatocytes can be isolated from donor livers, more than a billion cells are required per transplantation; hence we would either require many donor livers or would need to expand primary hepatocytes by culture to achieve the numbers of hepatocytes needed. Primary hepatocytes grown in tissue culture tend to dedifferentiate and lose most hepatic functions. Accordingly, the use of primary hepatocytes from donated livers for basic research and treatment has been unsatisfactory [23]. Producing hepatocytes using stem cells helps to avoid the need to purify primary hepatocytes from donor liv-

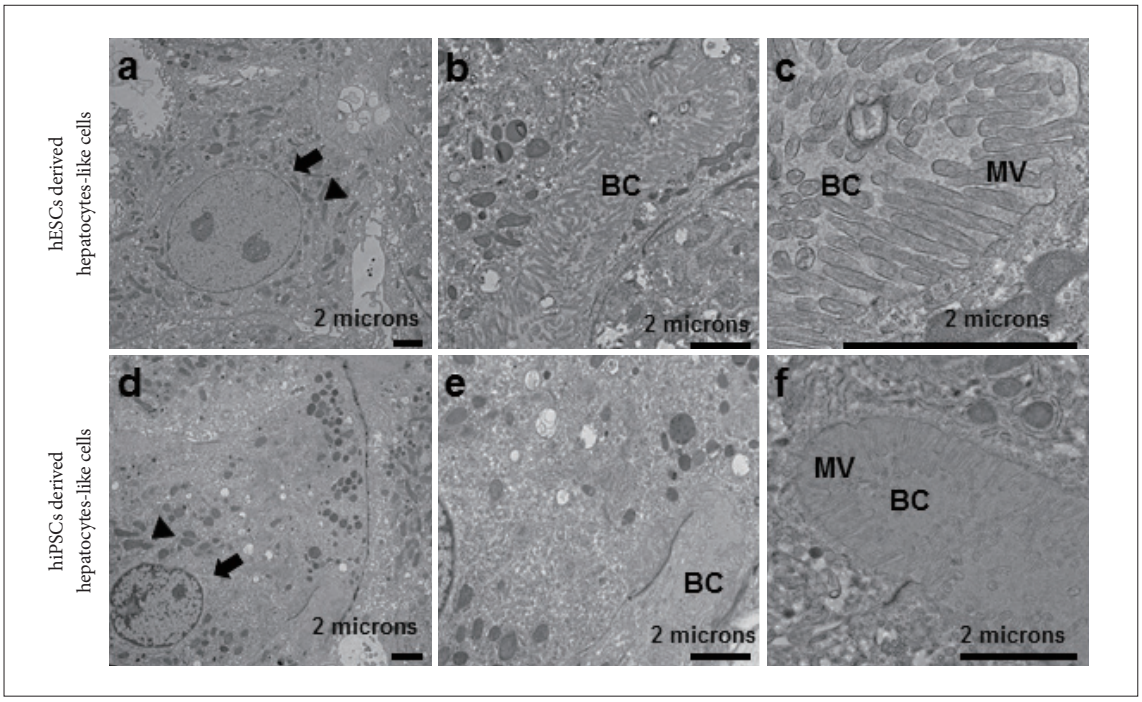

Figure 4. Visualization of HLCs by electron microscopy. hESC-derived HLCs (upper panel); hiPSCs-derived HLCs (lower panel). Large round euchromatic nuclei (arrows); numerous mitochondria (arrowheads). Magnifications: ×1000 (a, d); ×2500 (b, e); ×5000 (f); ×10000 (c). Scale bar, 2 microns. hESCs: human embryonic stem cells, hiPSCs: human induced pluripotent stem cells, HLCs: hepatocyte-like cells, BC: bile canaliculi, MW: abundant microvilli.

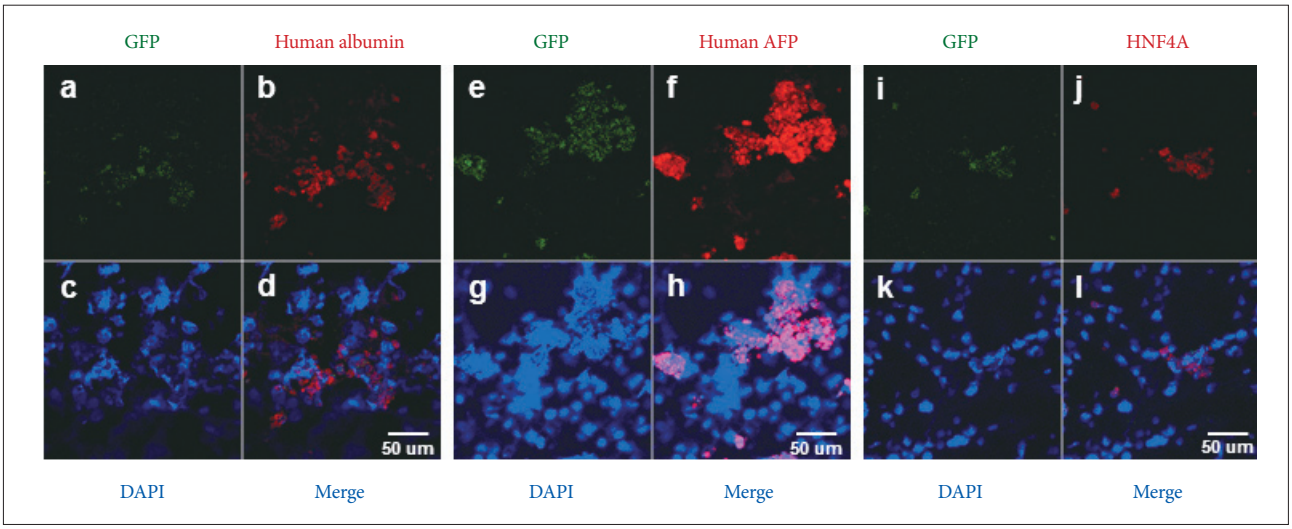

Figure 5. Transplantation of HLCs into MUP-uPA/SCID mouse. 10^6 cells/50 uL in HBSS were injected into the inferior poles of the spleen. GFP-expressing cells are located in liver (a, e, i), and GFP-expressing HLCs are staining with human albumin (b, d), AFP (f, h) and HNF4A (j, l). DAPI (c, d, g, h, k, l) labels all nuclei. Scale bar, 50 um. AFP: alpha-fetoprotein, DAPI: 4',6-diamidino-2-phenylindole, HLCs: hepatocyte-like cells, MUP-uPA/SCID: major urinary protein-urokinase-type plasminogen activator/severe combined immunodeficiency, HBSS: Hank's Balanced Salt Solution.

ers. Both ESCs and iPSCs have the ability to multiply without limit and without loss of potency. Many studies have shown that hESCs and hiPSCs can differentiate into HLCs [24-30]. Some researchers report single cell seeding methods and differentiation into HLCs [25-27]. They showed that SOX17 expression almost 80% in endodermal cells by immunostaining [26]. Our methods of EB formation also showed more than 80% SOX17 expression under fluorescence microscope (Fig. 1D). However, it could not be easy to compare the differentiation efficiency between these two studies because of different experimental systems. However, we think that the protocol used here has many advantages for generating HLCs. We have shown that sufficient HLCs can be generated from hESCs and hiPSCs.

Also whether iPSCs and ESC have similar or different gene expression profiles is unclear. Some researchers have reported only small differences in gene expression between hESCs and hiPSCs [31] but their methylation profiles were found to be different [32-34]. However, their differentiation potential was very similar [35] though hiPSCs were less efficient. To confirm this, we obtained HLCs from hESCs and hiPSCs, and compared their differentiation. The hepatocyte markers expressed during differentiation of the hiPSCs were very similar to those expressed by the hESCs. The functions of the two sets of differentiated cells were also very similar. These findings underline the similarity of functional properties of hESCs and hiPSCs. Therefore, we propose to generate HLCs from patients and normal individuals with the protocols we have developed and to examine their behavior when transplanted into a mouse liver transplant model that is a useful model of liver disease.

Supplementary Materials

The online-only Data Supplement is available with this article at https://doi.org/10.1007/s13770-016-0094-y.

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Conflicts of Interest

The authors have no financial conflicts of interest.

Ethical Statement

This research protocol was approved by the Hanyang University Institutional Review Board.

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