A Paracrine Mechanism Accelerating Expansion of Human Induced Pluripotent Stem Cell-Derived Hepatic Progenitor-Like Cells

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Hepatic stem/progenitor cells in liver development have a high proliferative potential and the ability to differentiate into both hepatocytes and cholangiocytes. In this study, we focused on the cell surface molecules of human induced pluripotent stem (iPS) cell-derived hepatic progenitor-like cells (HPCs) and analyzed how these molecules modulate expansion of these cells. Human iPS cells were differentiated into immature hepatic lineage cells by cytokines. In addition to hepatic progenitor markers (CD13 and CD133), the cells were coimmunostained for various cell surface markers (116 types). The cells were analyzed by flow cytometry and in vitro colony formation culture with feeder cells. Twenty types of cell surface molecules were highly expressed in CD13⁺CD133⁺ cells derived from human iPS cells. Of these molecules, CD221 (insulin-like growth factor receptor), which was expressed in CD13⁺CD133⁺ cells, was quickly downregulated after in vitro expansion. The proliferative ability was suppressed by a neutralizing antibody and specific inhibitor of CD221. Overexpression of CD221 increased colony-forming ability. We also found that inhibition of CD340 (erbB2) and CD266 (fibroblast growth factor-inducible 14) signals suppressed proliferation. In addition, both insulin-like growth factor (a ligand of CD221) and tumor necrosis factor-like weak inducer of apoptosis (a ligand of CD266) were provided by feeder cells in our culture system. This study revealed the expression profiles of cell surface molecules in human iPS cell-derived HPCs and that the paracrine interactions between HPCs and other cells through specific receptors are important for proliferation.

Introduction

THE LIVER IS THE LARGEST and a vital organ in mammals. It performs many important functions, such as metabolism, plasma protein synthesis, glycogen storage, bile secretion, and detoxification. Decompensated chronic and acute liver diseases such as cirrhosis or fulminant hepatic failure are life-threatening conditions, and liver transplantation is the effective treatment of choice. However, this approach is limited because of a shortage of donor organs, and many patients do not survive while waiting for a liver transplantation. Transplantation of hepatocytes or stem/progenitor cells into the liver may serve as an alternative treatment [1]. Furthermore, an established differentiation protocol to obtain functional hepatocytes from human pluripotent stem cells can be applied to drug discovery, toxicology testing, and disease modeling such as viral hepatitis and inherited liver diseases [2–5]. Human induced pluripotent stem (iPS) cells are derived from somatic stem cells using Yamanaka factors [ie,

Oct3/4, SRY-related HMG box (SOX)2, and Kruppel-like factor (Klf)4]. These cells possess a self-renewal ability and pluripotency to differentiate into all cell types of the three primary germ layers: ectoderm, mesoderm, and endoderm [6]. Therefore, functional human iPS cell-derived cells, such as hepatocytes, are considered to be a potentially good source for cell therapies, particularly to avoid ethical issues and limit immunogenicity [7,8]. A large number of functional human iPS cell-derived hepatocytes are required for regenerative cell therapy of the liver as well as drug development. Expansion of mature hepatocytes *ex vivo* is very difficult, whereas hepatic progenitor-like cells (HPCs) have a high proliferative potential [9]. To obtain a large number of functional human iPS cell-derived hepatocytes, HPCs are likely to be useful. After purification and expansion of HPCs from iPS cell cultures, the HPCs can be induced to differentiate into mature hepatocytes.

Differentiation of human iPS cells into hepatocytic cells is induced by serial addition of cytokines and growth

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factors, which mimics *in vivo* developmental processes. Liver organogenesis begins at early embryonic stages from the foregut endoderm that is stimulated by fibroblast growth factor (FGF) from the adjacent cardiac region [10] and bone morphogenetic protein (BMP) from the septum transversum mesenchyme [11]. Subsequently, these cells commit to hepatoblasts (fetal hepatic progenitor cells) and form the liver bud with hepatocyte growth factor (HGF) involved in this hepatoblast expansion step. The cytokines and growth factors expressed in the developing liver can induce hepatoblasts from human iPS cells [12–15]. The expansion and differentiation of HPCs are regulated by interactions with other cell types such as mesenchymal cells in fetal livers. For example, several mesenchymal cell-related transcription factors (eg, Hlx and Lhx2) are strictly required for fetal liver development [16,17]. To mimic the interaction between hepatic and mesenchymal cells, we previously reported that a coculture system with mouse embryonic fibroblasts (MEFs) has the ability to support the proliferation of mouse hepatoblasts and human iPS cell-derived HPCs *in vitro* [15,18]. It has also been reported that human pluripotent stem cell-derived HPCs can be maintained on STO feeder cells [19] and laminin 111-coated dishes [14]. Therefore, the paracrine interactions with extrinsic factors (eg, extracellular matrices, feeder cells, and growth factors produced by feeder cells) are important to maintain the proliferation potency of HPCs. However, the cell surface molecules required for the interaction with these extrinsic factors remain unknown.

Cell surface molecules are useful for the identification and characterization of cells. CD13 and CD133 are mouse hepatoblast-specific cell surface molecules during the early and intermediate (embryonic days 9.5–14) stages of fetal development [18,20]. We previously found that HPCs are enriched in the CD13⁺CD133⁺ fraction of cells differentiated from human iPS cells. These cells have a high proliferation potential, the ability to differentiate into both hepatocytes and cholangiocytes, and expand in long-term culture [15]. In the present study, we focused on the correlation between cell surface molecules and the characteristics of human iPS cell-derived HPCs and revealed the expansion mechanisms of human iPS cell-derived HPCs by identifying highly expressed cell surface receptors.

Materials and Methods

Human iPS cell culture

A human iPS cell line, TkDA3-4, was established as described previously [21]. Some of the experiments were performed using a human iPS cell line, RIKEN-2F, derived from Riken BioResource Center (Tsukuba, Japan) [22]. The study was conducted in accordance with the Declaration of Helsinki. Human iPS cells were maintained according to standard methods [21]. Briefly, iPS cells were cultured on mitomycin C (MMC, Wako Pure Chemical Industries Ltd., Osaka, Japan)-treated MEF cells as feeder cells in Dulbecco's modified Eagle's medium/F-12 medium (DMEM/F-12; Sigma, St. Louis, MO) supplemented with 0.1 mM nonessential amino acids (Life Technologies, Carlsbad, CA), 20% knockout serum replacement (Life Technologies), 0.1 mM 2-mercaptoethanol, and 5 ng/mL basic FGF (bFGF; Wako Pure Chemical Industries Ltd.). MMC-treated MEFs were

established according to standard methods [15]. iPS cells were passaged every 5–7 days to maintain their undifferentiated state.

Differentiation of human iPS cells toward hepatic lineage cells *in vitro*

The differentiation protocol for induction of hepatic lineage cells was based on our previous report [15] with some modifications. Differentiation of iPS cells into definitive endodermal cells was induced by a 3-day activin A differentiation protocol [23]. Cells were incubated with 100 ng/mL recombinant human activin A (PeproTech, Rocky, NJ) in RPMI 1640 (Sigma) supplemented with increasing concentrations of B27 medium supplement (B27; Life Technologies) (0%, days 1–2; 0.2%, days 1–2; 2%, days 2–3). After 3 days of endodermal differentiation, the cells were incubated at days 4–7 in RPMI 1640 with 2% B27, 10 ng/mL bFGF, and 20 ng/mL recombinant human BMP-4 (PeproTech). Then, the cells were incubated at days 8–11 in RPMI 1640 with 2% B27 and 40 ng/mL recombinant human HGF (PeproTech).

Flow cytometry

Cells were trypsinized using 0.05% trypsin–ethylenediaminetetraacetic acid (Sigma). Trypsinized cells were washed with phosphate-buffered saline (PBS) containing 3% fetal bovine serum (FBS), and then incubated with antibodies against cell surface proteins (shown in Table 1; Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/scd) for 1 h at 4°C. After washing with PBS containing 3% FBS and staining the dead cells with propidium iodide, the cells were analyzed and sorted by fluorescence-activated cell sorting using FACSARIA

TABLE 1. FLOW CYTOMETRIC ANALYSES OF THE HUMAN INDUCED PLURIPOTENT STEM CELL-DERIVED CD13⁺ CD133⁺ FRACTION

<i>Positive</i>	<i>Split</i>	<i>Barely positive</i>		<i>Negative</i>	
(> 85%)	(40%–85%)	(5%–40%)		(< 5%)	
CD24	CD9 ^a	CD10	CD150	CD14	CD206
CD26	CD54 ^a	CD27 ^a	CD154 ^a	CD15	CD207
CD47	CD55 ^a	CD28 ^a	CD161 ^a	CD19	CD226
CD58	CD68 ^a	CD43 ^a	CD162	CD30	CD258
CD63	CD70 ^a	CD48 ^a	CD170	CD36	CD261
CD81	CD82 ^a	CD52 ^a	CD180	CD38	CD267
CD99	CD86 ^a	CD53 ^a	CD200R	CD39	CD268
CD112	CD90 ^a	CD62P	CD201 ^a	CD40	CD271
CD147	CD105 ^a	CD73	CD202b ^a	CD44	CD273
CD156c	CD111 ^a	CD74 ^a	CD205	CD50	CD304
CD166	CD135 ^a	CD80	CD209	CD56	CD328
CD221	CD143 ^a	CD83	CD220	CD62E	CD334
CD262	CD146 ^a	CD87 ^a	CD243 ^a	CD62L	CD338
CD266	CD157 ^a	CD88	CD244	CD66a/c/e	
CD276	CD172a/b ^a	CD89	CD254 ^a	CD93	
CD321	CD231 ^a	CD96	CD255 ^a	CD117	
CD324	CD277 ^a	CD97 ^a	CD257	CD125	
CD325	CD344 ^a	CD102 ^a	CD263 ^a	CD140a	
CD326		CD107a ^a	CD279	CD167a	
CD340		CD134 ^a	CD294	CD169	
		CD137 ^a	CD303	CD177	
		CD138		C203c	

^aCell surface molecules used for colony formation assays.

(BD Biosciences, San Jose, CA). Data analysis was performed using FlowJo (Tree Star, Inc., Ashland, OR).

Transcription profile analysis using microarrays

CD13⁺CD133⁺ HPCs derived from human iPS cells were purified as described above. Total RNA was purified from these cells using the RNeasy Micro Kit (Qiagen, Victoria, Australia) according to the manufacturer's instructions. Transcription profiles were analyzed using the Agilent Whole Human Genome Microarray 4×44K. The original data are available from Gene Expression Omnibus (accession number GSE66450). Expression data were analyzed using Gene Springs. Datasets were normalized; CD antigen genes were extracted and have been represented as a heat map.

Colony formation assay

Human iPS cell-derived HPC culture and passaging were based on our previous report [15]. Briefly, MMC-treated MEFs (2×10⁵ cells/well) were seeded in 0.1% gelatin (from porcine skin; Sigma)-coated 12-well plates the day before sorting. Sorted cells were plated onto an MEF feeder layer at 1,000 or 1,500 cells per well. The standard culture medium was a 1:1 mixture of hepatic-colony-forming units in culture (H-CFU-C) medium and DMEM/10% FBS (Nichirei Biosciences, Tokyo, Japan). H-CFU-C medium consisted of DMEM/F-12 supplemented with 10% FBS (Nichirei Biosciences), 1×insulin–transferrin–selenium X (Life Technologies), 10 mM nicotinamide (Sigma), 10^{−7} M dexamethasone (Sigma), 2.5 mM HEPES buffer solution (Life Technologies), 1×penicillin–streptomycin–glutamine, and 1×non-essential amino acids. For expansion, the cells were cultured in standard medium supplemented with 0.25 μM A-83-01 (Wako Pure Chemical Industries Ltd.), 10 μM Y-27632 (Wako Pure Chemical Industries Ltd.), 40 ng/mL HGF, and 20 ng/mL recombinant human epidermal growth factor (EGF; PeproTech). The medium was replaced every 3 days.

To stimulate the effect of erbB receptor signaling, HPCs were incubated with or without recombinant human EGF or 50 ng/mL recombinant human Neuregulin 1 (heregulin)-β1 (NRG1; PeproTech) immediately after plating. To stimulate the effect of tumor necrosis factor-like weak inducer of apoptosis (TWEAK)/fibroblast growth factor-inducible 14 (Fn14) signaling, HPCs were incubated with 25 or 50 ng/mL recombinant human TWEAK (BioLegend, San Diego, CA) immediately after plating. To stimulate the effect of insulin-like growth factor (IGF)-1 receptor (IGF-1R) signaling, HPCs were incubated with 100 ng/mL IGF-1 (PeproTech) or 100 ng/mL IGF-2 (PeproTech) immediately after plating in the absence of insulin–transferrin–selenium X. To block the effect of erbB receptor signaling, cells were incubated with the erbB2 inhibitor, AG825 (5 μM; Cayman Chemical, Ann Arbor, MI), 1 day after plating. To block the effect of TWEAK-Fn14 signaling, cells were incubated with an anti-human Fn14 blocking antibody (2 μg/mL, clone ITEM-4, functional grade purified; eBioscience, Santa Clara, CA) or mouse IgG2b kappa isotype control antibody (clone eBMG2b, functional grade purified; eBioscience) 1 day after plating. To block the effect of IGF-1R signaling, cells were incubated with an anti-human IGF-1R blocking antibody (5 μg/mL, clone 1H7; LEAFTM purified, BioLegend) or mouse IgG1 kappa isotype control antibody (clone MG1-45;

LEAFTM purified, BioLegend) 1 day after plating. We also used an IGF-1R-specific inhibitor, PQ401 (10 μM; Sigma).

Immunocytochemistry

Cultured cells were washed with PBS and fixed with 4% paraformaldehyde (Wako Pure Chemical Industries, Ltd./PBS for 15 min at room temperature. After washing with PBS, the cells were incubated in 0.5% Triton/PBS for 10 min at room temperature. After washing with PBS, the cells were incubated with 5% donkey serum (Millipore, Bedford, MA) in PBS for 1 h at room temperature. Then, the cells were incubated overnight with diluted primary antibodies at 4°C. A rabbit anti-alpha-fetoprotein (AFP) antibody (Dako, Glostrup, Denmark) and goat anti-hepatocyte nuclear factor 4 alpha (HNF4α) antibody (Santa Cruz Biotechnology, Santa Cruz, CA) were used as primary antibodies. The cells were washed with PBS several times and then incubated with diluted secondary antibodies for 1 h at room temperature. Anti-rabbit IgG-Alexa 568 (Life Technologies) and anti-goat IgG-Alexa 488 (Life Technologies) were used as secondary antibodies. The cells were washed with PBS, and nuclei were stained with 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI; Sigma).

After immunocytochemistry, single cell-derived colonies were measured with a Cellomics ArrayScan VTI HCS Reader (Thermo Fisher Scientific, Waltham, MA). Colonies were recognized by AFP and HNF4α expression, and the number of cells in individual colonies was counted based on DAPI staining. Data analyses were performed using Cello-micsR view 1.6.3.3 (Thermo Fisher Scientific).

RNA isolation and real-time polymerase chain reaction

To analyze gene expression, total RNA from CD13⁺CD133⁺ iPS cell-derived HPCs, MMC-treated MEFs (at 0, 2, and 6 days of culture), NIH3T3 cells, and E12 mouse whole embryo cells was purified with an RNeasy Micro Kit (Qiagen). cDNAs were synthesized using a High-Capacity cDNA Reverse Transcription Kit (Life Technologies) according to the manufacturer's instructions. Real-time polymerase chain reaction (PCR) was performed with the Universal Probe Library (Roche Diagnostics, Basel, Switzerland) using a StepOnePlus Real-Time PCR system (Life Technologies). Gene expression was normalized to the expression of beta-2 microglobulin that served as a reference gene in undifferentiated cells [24] or hypoxanthine phosphoribosyltransferase 1 (HPRT1) that served as a housekeeping gene. Primer sequences and probe numbers for each gene are listed in Supplementary Table S2. Data were quantified with StepOnePlus software v2.2.1 (Life Technologies).

IGF-1R overexpression in human iPS cells

Human *IGF-1R* cDNA was amplified by PCR and ligated into a pENTR-1A gateway entry vector (Life Technologies). The human *IGF-1R* vector (pBPV-IGF-1R, kindly provided by Prof. Masahiko Miura, Tokyo Medical and Dental University) was used as a template for PCR [25]. Then, IGF1-R cDNA was ligated into a piggyBac expression vector using the Gateway system (Life Technologies).

To establish constitutively *IGF-1R*-expressing human iPS cells, the human *IGF-1R* expression piggyBac vector and PBase expression vector were cotransfected into TkDA3-4 cells using the Neon transfection system (Life Technologies) and selected with G418. After 4 days of G418 selection, surviving cells were used as constitutively *IGF-1R*-expressing human iPS cells.

Statistical analyses

Calculations of statistically significant differences between samples using the Student's two-tailed *t*-test as well as the standard deviation were performed using the Microsoft Excel 2013 software.

Results

Analyses of progenitor-like cell phenotypes in cultures of hepatic cells differentiated from human iPS cells

Serial cytokine stimulation induces the differentiation of the human iPS cell line, TkDA3-4, into definitive endodermal, hepatic endodermal, and immature hepatocytic cells in vitro (Fig. 1A). Previously, we found that the CD13⁺CD133⁺ fraction of iPS cell-derived cells contains HPCs with a high proliferative ability and potency to differentiate into both hepatic and cholangiocytic progenitor cells [15]. However, the characteristics of this HPC fraction remain unknown. The biological functions of progenitor and mature

FIG. 1. Flow cytometric analyses of progenitor-like cells in hepatic cell cultures differentiated from human iPS cells. **(A)** Schematic representation of the protocol for differentiation of human iPS cells toward hepatic lineage cells. HPCs were isolated by fluorescence-activated cell sorting. HPCs were enriched in the CD13⁺CD133⁺ cell fraction. **(B)** Flow cytometric analyses of the CD13⁺CD133⁺ fraction and criteria for categorization according to the ratio of positive cells for cell surface molecules. Representative gates for the CD13⁺CD133⁺ fraction are shown in the left panel. Histograms of each category are shown in right panels. Blue, isotype control; red, stained with the antibody against the cell surface molecule. iPS, induced pluripotent stem; HPC, hepatic progenitor-like cell.

cells are regulated by both several soluble factors derived from other cells and the cell–cell interactions. Cell surface receptors and antigens are important for these interactions. Therefore, we comprehensively analyzed the expression of cell surface molecules using specific antibodies. After differentiation of human iPS cells, dissociated cells were stained with anti-CD13 and -CD133 antibodies and one other antibody against various cell surface molecules (116 types, as shown in Supplementary Table S1). Then, the cells were analyzed by flow cytometry. According to the ratio of the positive cell population in the CD13⁺CD133⁺ fraction, cell surface molecules were classified into four categories, positive (>85%), split (40%–85%), barely positive (5%–40%), and negative (<5%) (Fig. 1B). Results are summarized in Table 1. Twenty types of cell surface molecules were highly expressed in the CD13⁺CD133⁺ fraction of differentiated human iPS cells (Positive in Table 1). These cell surface molecules were expressed in hepatic progenitor cells derived from another human iPS cell line, RIKEN-2F (Supplementary Fig. S1). In addition, the CD13⁺CD133⁺ fraction had differential expression of various cell surface molecules (Split and Barely positive in Table 1). Next, we compared these antibody screening results of cell surface molecules with the mRNA expression results (Supplementary Fig. S2). The majority of cell surface molecules categorized as negative using flow cytometry were barely detected by microarray expression analysis. Consequently, the CD13⁺CD133⁺ fraction was heterogeneous with regard to these surface molecules.

Correlation between the expression of the cell surface molecules and colony-forming activity

We sorted CD13⁺CD133⁺ iPS cell-derived HPCs by the expression level of cell surface molecules that were heterogeneously expressed in this population (Split and Barely positive molecules indicated by the superscript a in Table 1). We compared the proliferative abilities of these subpopulations in a colony formation assay. In the analyses of these 39 split and barely positive molecules, both positive and negative fractions for these surface molecules formed large colonies under our culture conditions. For example, the expression levels of CD54, CD90, CD146, and CD231 were not associated with the proliferative ability of HPCs (Supplementary Fig. S3). These data suggest that CD13 and CD133 are appropriate cell surface molecules to enrich HPCs from differentiated iPS cells with a high proliferative potential.

Suppression of cell surface molecules of human iPS cell-derived HPCs during long-term culture

As shown above, we found 20 positive cell surface molecules expressed on human iPS cell-derived CD13⁺CD133⁺ HPCs. These cells have the ability to expand in cocultures with MEF feeder cells [15]. Next, we analyzed the changes in expression of these cell surface molecules during long-term culture in vitro (Fig. 2A). CD221 (IGF-1R) and CD325 (N-cadherin) expression was downregulated (Fig. 2B, C) after in vitro expansion. In contrast, there was barely any change in the expression of other molecules (Supplementary Fig. S4). Both positive and negative cells

for CD221 and CD325 after the first culture step were sorted and their proliferative ability was analyzed in the colony formation assay. Compared with CD221[−] cells, CD221^{high} cells had a high proliferative ability. In contrast, the proliferative ability of CD325^{high} and CD325[−] cells was not significantly different (Fig. 2D). Expression of CD221 mRNA in CD13⁺CD133⁺ cells was also downregulated after the first culture (Fig. 2E). These results suggest that CD13⁺CD133⁺ CD221^{high} cells, which have a more progenitor-like potential and high proliferative ability, are retained in our in vitro culture system.

IGF-1R signaling in human iPS cell-derived HPCs

CD221, also known as IGF-1R, is a transmembrane tyrosine kinase receptor that is activated by its specific ligands, including IGF-1 and IGF-2. Because the CD221^{high} fraction had an efficient proliferative ability, we next analyzed the effects of IGF-1R signaling in human iPS cell-derived HPCs. The addition of an anti-human IGF-1R blocking antibody significantly suppressed the colony-forming activity (Fig. 3A). Similarly, the addition of PQ401, a specific inhibitor of IGF-1R, also suppressed the colony-forming activity (Fig. 3B). Unexpectedly, the addition of recombinant IGF-1 or IGF-2 to the culture medium did not significantly improve HPC colony formation (Fig. 3C). Because our culture system used MEFs as feeder cells, we analyzed the gene expression of IGF-1 and IGF-2 in MEFs. As a result, we found that IGF-1 and IGF-2 were expressed in MEFs, but not in NIH3T3 cells, which were used as the negative control (Fig. 3D). These results indicated that paracrine signaling activated IGF-1R through IGF-1 and IGF-2 from feeder cells, which contributed to the cell proliferation of human iPS-derived HPCs.

Next, we established human iPS cells with an IGF-1R constitutive expression cassette using the piggyBac transposon system and differentiated these cells to hepatic lineage cells. As shown in Fig. 3E, CD221 was expressed in both mock and CD221-overexpressing human iPS cells (undifferentiated). After differentiation into HPCs by serial cytokine addition, CD221 was also expressed in CD13⁺CD133⁺ HPCs derived from mock and CD221-overexpressing human iPS cells. After the first and second cultures, the expression level of CD221 in the CD13⁺CD133⁺ fraction derived from CD221-overexpressing iPS cells was higher than that of cells derived from mock iPS cells (Fig. 3E, F, P1 and P2 cells). We performed colony formation assays using P0 CD13⁺CD133⁺ HPCs derived from both mock and CD221-overexpressing iPS cells. The number of large colonies was higher in CD221-overexpressing CD13⁺CD133⁺ cells than in mock cells (Fig. 3G), suggesting that maintenance of CD221 expression is important for the proliferation function of progenitor cells.

ErbB receptor signaling in human iPS cell-derived HPCs

As shown above, CD340 (erbB2), a member of the erbB transmembrane tyrosine kinase receptor family, was also highly expressed on CD13⁺CD133⁺ HPCs. The erbB family consists of the epidermal growth factor receptor (EGFR), erbB2 (CD340/HER2/neu), erbB3 (HER3), and

FIG. 2. Expression changes of cell surface molecules on human iPS cell-derived HPCs during long-term culture. **(A)** Schematic representation of the culture steps during long-term culture. After 11 days of cytokine stimulation, P0 CD13⁺CD133⁺ cells derived from the human iPS cell culture were purified using a flow cytometer and cultured on MEFs. After 14 days of culture (first culture), cells were dissociated and P1 CD13⁺CD133⁺ cells were analyzed and purified. After inoculation of these P1 cells on new MEFs, cells were also cultured for 14 days (second culture). P2 CD13⁺CD133⁺ cells were then analyzed. **(B)** Expression of CD221 and CD325 in the CD13⁺CD133⁺ fraction during long-term culture. Expression of CD221 and CD325 was high in the primary CD13⁺CD133⁺ fraction and downregulated after in vitro expansion (shown as the P1 and P2 CD13⁺CD133⁺ fractions). White, isotype control; gray, stained with the antibody against the cell surface molecule. Expression of the isotype control set the gate in approximately <1%. Representative data are shown (two independent experiments). **(C)** MFI for PE-CD221 and PE-CD325 on P0, P1, and P2 CD13⁺CD133⁺ cells shown in **(B)**. MFI was normalized to the PE-isotype control that was calculated for each assay. **(D)** Colony formation assays and the numbers of large colonies (consisting of over 100 cells) derived from 1,000 sorted cells. Cells positive and negative for these surface markers in the P1 CD13⁺CD133⁺ fraction were sorted and analyzed by colony formation culture. CD221⁻ (gate i), CD221^{high} (gate ii), CD325⁻ (gate i), and CD325^{high} (gate ii) cells in the CD13⁺CD133⁺ fraction were cultured (gates are shown in histograms). Results are represented as the mean colony count $\pm$ SD (triplicate culture samples). * P < 0.01. Representative data are shown (two independent experiments). **(E)** The expression levels of IGF-1R, N-cadherin, erbB2, and Fn14 were measured in P0 and P1 CD13⁺CD133⁺ cell fractions by quantitative real-time PCR analyses. Gene expression was normalized to the expression of beta-2 microglobulin. The expression of genes in P0 CD13⁺CD133⁺ cells was set to 1.0. Values are the mean $\pm$ SD from two independent experimental samples. MEF, mouse embryonic fibroblast; MFI, mean fluorescence intensity; IGF-1R, IGF-1 receptor; Fn14, fibroblast growth factor-inducible 14; SD, standard deviation; PCR, polymerase chain reaction.

FIG. 3. Effect of IGF-1R signaling in human iPS cell-derived HPCs. **(A)** P0 CD13⁺CD133⁺ HPCs derived from human iPS cells were cultured with or without the anti-human IGF-1R blocking antibody. An isotype antibody was used for the negative control. **(B)** Colony formation assay derived from P0 CD13⁺CD133⁺ HPCs in the presence of the IGF-1R-specific inhibitor. Cells were incubated with PQ401. **(C)** Colony formation assay derived from P0 CD13⁺CD133⁺ HPCs in the presence of IGF-1R-specific ligands. Cells were incubated with IGF-1 or IGF-2 in the absence of insulin. **(A–C)** The panels show the numbers of large colonies (consisting of over 200 cells) derived from 1,500 sorted cells. Results are represented as the mean colony count $\pm$ SD (triplicate culture samples). * P < 0.05; ** P < 0.01. Representative data are shown (two independent experiments). **(D)** The expression levels of IGF-1 and IGF-2 were measured in MEFs (after 0, 2, and 6 days of culture), NIH3T3 cells, and mouse E12 whole embryo (E12WE) cells by quantitative real-time PCR analyses. Gene expression was normalized to the expression of a housekeeping gene (HPRT1). The expression of genes in E12 whole embryo cells was set to 1.0. Values are the mean $\pm$ SD from two independent experimental samples. **(E)** Flow cytometric analysis of CD221 expression on human iPS cells (mock) and CD221-overexpressing human iPS cell-derived cells. Human iPS cells, P0, P1, and P2 CD13⁺CD133⁺ cells, were analyzed by flow cytometry. Expression of the isotype control set the gate in approximately < 1%. White, isotype control; gray, CD221. Representative data are shown (two independent experiments). **(F)** MFI for PE-CD221 on human iPS, P0, P1, and P2 CD13⁺CD133⁺ cells, derived from **(D)**. MFI was normalized to the PE-isotype control that was calculated for each assay. **(G)** Colony formation assays of P0 CD13⁺CD133⁺ HPCs derived from mock and CD221-overexpressing human iPS cells. The panel shows the numbers of large colonies (consisting of over 200 cells) derived from 1,000 sorted cells. Results are represented as the mean colony count $\pm$ SD (triplicate culture samples). * P < 0.05. Representative data are shown (two independent experiments). IGF, insulin-like growth factor; HPRT1, hypoxanthine phosphoribosyltransferase 1.

FIG. 4. Effect of erbB receptors and Fn14 signaling in human iPS cell-derived HPCs. **(A)** Histograms of erbB family expression (EGFR, CD340/erbB2, erbB3, and erbB4) on P0 CD13⁺CD133⁺ HPCs derived from human iPS cells. *white*, isotype control; *gray*, stained with the antibody against the cell surface molecule. Expression of the isotype control set the gate in approximately <1%. Representative data are shown (two independent experiments). **(B)** Effect of EGF and NRG1 on the expansion of human iPS cell-derived HPCs. P0 CD13⁺CD133⁺ HPCs were incubated without EGF, with NRG1 only, EGF only (standard culture medium), or EGF plus NRG1. **(C)** Effect of a specific inhibitor of erbB2 on the expansion of human iPS cell-derived HPCs. P0 CD13⁺CD133⁺ HPCs were incubated with AG825. **(B, C)** The panels show the numbers of large colonies (consisting of over 200 cells) derived from 1,500 sorted cells. Results are represented as the mean colony count $\pm$ SD (triplicate culture samples). * P < 0.05. Representative data are shown (two independent experiments). **(D)** Expression levels of EGF, NRG1, and TWEAK were measured in MEFs (after 0, 2, and 6 days of culture), NIH3T3 cells, and mouse E12 whole embryo (E12WE) cells by quantitative real-time PCR analyses. Gene expression was normalized to the expression of a housekeeping gene (HPRT1). The expression of genes in WE12 whole embryo cells was set to 1.0. Values are the mean $\pm$ SD from two independent experimental samples. ND indicates not detected. **(E)** Effect of an Fn14 blocking antibody on the expansion of human iPS cell-derived HPCs. P0 CD13⁺CD133⁺ HPCs were cultured with an anti-human Fn14 blocking antibody. **(F)** Effect of an Fn14 ligand on expansion of human iPS cell-derived HPCs. P0 CD13⁺CD133⁺ HPCs were cultured with recombinant human TWEAK. **(E, F)** The panels show the numbers of large colonies (consisting of over 200 cells) derived from 1,500 sorted cells. Results are represented as the mean colony count $\pm$ SD (triplicate culture samples). ** P < 0.01. Representative data are shown (two independent experiments). EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; NRG1, neuregulin 1; TWEAK, tumor necrosis factor-like weak inducer of apoptosis.

erbB4 (HER4) [26]. In response to ligand binding, erbB protein undergoes dimerization, leading to activation of erbB tyrosine kinase domains and phosphorylation of the tyrosine residues in the C-terminal tail. This activation subsequently induces recruitment and activation of downstream cellular signaling proteins [27]. Because erbB2 is considered to be a preferred heterodimerization partner of

other erbB receptors and important for their receptor signaling [28,29], we investigated the expression of EGFR, erbB3, and erbB4 on CD13⁺CD133⁺ human iPS cell-derived HPCs by flow cytometry. As a result, we found high expression of both EGFR and erbB2. In contrast, we found modest expression of erbB3 and no expression of erbB4 on CD13⁺CD133⁺ cells (Fig. 4A).

To investigate whether erbB signaling plays a role in the proliferation of CD13⁺CD133⁺ human iPS cell-derived HPCs, we performed colony formation assays in the presence or absence of EGF (a ligand of EGFR) and NRG1 (a ligand of erbB3 and erbB4) [30]. Under our culture conditions, the colony formation activity of iPS cell-derived HPCs was markedly suppressed without EGF. However, the colony formation activity was slightly induced by NRG1 in cultures without EGF. The colony formation activity was significantly induced by addition of both EGF and NRG1 (Fig. 4B). In addition, an inhibitor of erbB2 (AG825) suppressed the colony-forming activity of iPS cell-derived HPCs (Fig. 4C). We also analyzed the gene expression of EGF and NRG1 in MEF feeder cells. The expression of EGF and NRG1 was substantially low in MEFs compared with that in mouse whole embryo E12 cells (Fig. 4D), indicating that EGF and NRG1 were not supplied by feeder cells. These results suggested that erbB family proteins, such as EGFR, erbB2, and erbB3, contribute to the cell proliferation of CD13⁺CD133⁺ iPS-derived HPCs.

Fn14 signaling in human iPS cell-derived HPCs

CD266, also known as Fn14, was highly expressed on CD13⁺CD133⁺ human iPS-derived HPCs. It is known that Fn14 is a member of the tumor necrosis factor receptor superfamily and TWEAK is its ligand [31]. We analyzed the role of TWEAK-Fn14 signaling in the proliferation of iPS cell-derived HPCs using colony formation assays. The addition of an anti-human Fn14 blocking antibody significantly suppressed the colony-forming activity (Fig. 4E). Next, we analyzed the gene expression of TWEAK in MEF feeder cells and found that TWEAK was highly expressed in MEFs (Fig. 4D), whereas the colony-forming activity was barely changed by the addition of TWEAK to the cultures (Fig. 4F). These results suggested that TWEAK, which is supplied by feeder cells, and Fn14 signaling contribute to the cell proliferation of CD13⁺CD133⁺ human iPS cell-derived HPCs.

As shown above, IGF-1, IGF-2, NRG1, and TWEAK were considered to be involved in proliferation of human iPS cell-derived HPCs. Next, we analyzed which of these factors affected the differentiation of HPCs. When these factors were added into our colony formation culture, the expression of hepatic markers (AFP and HNF4 α) was detected in colonies formed from both control and factor-stimulated cultures (Supplementary Fig. S5).

Discussion

Cell surface molecules are useful for identification, isolation, and characterization of a particular type of cell. We previously showed that positive selection using the combination of anti-CD13 and CD133 antibodies is useful to isolate human iPS cell-derived HPCs [15]. In this study, we identified the comprehensive expression profiles of cell surface molecules in CD13⁺CD133⁺ HPCs using a lot of specific antibodies. Cell isolation for flow cytometry using enzymes sometimes causes downregulation of cell surface antigens. A few of the cell surface molecules (such as CD50, CD140a, and CD334) were detected by microarray, but not by flow cytometry. However, the majority of negative categorized cell surface antigens were not expressed at both mRNA and protein levels. Thus, these results may lead us to

understand the properties and functions of HPCs derived from human iPS cells.

It has been previously found that the human liver contains two types of multipotent progenitors, hepatic stem cells (HpSCs) and hepatoblasts [32,33]. HpSCs are CD54⁻CD56⁺ cells, whereas hepatoblasts are CD54⁺CD56⁻ cells. In CD13⁺CD133⁺ human iPS cell-derived HPCs, CD56 was not expressed (Negative in Table 1), CD54 was substantially expressed (Split in Table 1), and expression of CD54 and CD56 did not change during long-term culture (data not shown). These results suggested that these cells maintain hepatoblast-like phenotypes in the human liver during in vitro culture.

Our data revealed that 20 types of cell surface molecules were highly expressed on CD13⁺CD133⁺ HPCs. Some of these cell surface molecules (CD24 [34–36], CD26 [36,37], CD324 [32,36,38,39], CD325 [19], and CD326 [32,36,40]) are reported as common markers expressed on primary HPCs in human and mouse livers. It was previously described that CD90⁻CD117⁺CD34⁺ cells in human fetal livers presented hepatic progenitor characteristics [41,42]. In contrast, another study suggested that CD90⁺ [43] and CD117⁻ cells [36,44] are HPCs. In this study, CD13⁺CD133⁺ HPCs derived from human iPS cells were negative for CD117, and CD90, a marker of oval cells [45], was heterogeneously expressed. In addition, compared with previous comprehensive analyses of cell surface molecules on HPCs in the mouse fetal liver [20,46], CD73 was positive on mouse HPCs, but barely positive on HPCs derived from human iPS cells. Thus, the expression of several cell surface molecules in hepatic progenitor cells is controversial and might differ between mouse and human HPCs. Our data showed that several cell surface markers were heterogeneously expressed on the CD13⁺CD133⁺ fraction. However, there was almost no difference between these marker-positive and marker-negative cells in colony formation assays. Therefore, CD13 and CD133 are likely to be sufficient to purify the hepatic progenitor fraction in differentiated human iPS cells.

Among the analyses of cell surface receptors that were highly expressed on CD13⁺CD133⁺ HPCs, we found that CD221 (IGF-1R) contributed to the proliferation of HPCs. Human embryonic stem cells express IGF-1R and can be differentiated to fibroblast-like cells through suppression of IGF-1R [47]. During liver development, IGF-1R protein is strongly expressed in the fetus, but weakly in the adult [48]. In addition, Nanog-positive hepatocellular carcinoma shows upregulation of IGF-1R expression and maintenance of self-renewal through the IGF-1R signaling pathway [49]. These previous findings support that IGF-1R-positive cells have an immature function and the high proliferative ability of stem and progenitor cells.

We further demonstrated that the CD13⁺CD133⁺ HPC fraction of differentiated human iPS cells expressed erbB2 (CD340). Although erbB2 can homodimerize into a ligandless complex when overexpressed in cells, including various types of cancer cells, the colony formation activity was markedly suppressed in CD13⁺CD133⁺ HPCs without EGF and NRG1 treatment. Therefore, we confirmed that CD13⁺CD133⁺ cells did not exhibit an abnormal cell proliferation state. In view of the erbB receptor family, we showed that EGFR, erbB2, and erbB3 were expressed on CD13⁺CD133⁺ HPCs. Indeed, EGFR, erbB2, and erbB3 are

expressed during fetal and neonatal periods, but erbB2 disappears in the adult rat liver [50]. Moreover, we showed that Fn14 (CD266) was highly expressed on CD13⁺CD133⁺ HPCs. Several types of progenitor cells express Fn14, including mesenchymal stem cells [51], immature erythrocytes [52], osteoblasts [53], and neural lineage progenitors [54]. In a recent study, TWEAK-Fn14 signaling was found to activate the growth of liver progenitor cells expressing Fn14 during chronic liver injury [55–57] and partial hepatectomy [58] in mice. In agreement with these previous findings, human iPS cell-derived HPCs expressed Fn14, and TWEAK-Fn14 signaling contributed to their proliferative activity.

Taken together, we revealed the expression profiles of cell surface molecules on human iPS cell-derived CD13⁺CD133⁺ HPCs. These comprehensive expression profiles are useful information for the characterization of HPCs derived from human iPS cells in our differentiation protocol. Several studies showed that proliferative progenitor cells exist in primary liver tissues and the human iPS or embryonic stem cell-derived culture [19,32,33,36,41,43–45,59]. However, common characteristics between these progenitor cells remain unidentified. In this study, we demonstrated that IGF-1R, EGFR, erbB2, and Fn14 were highly expressed and were involved in the proliferation of CD13⁺CD133⁺ HPCs, indicating that maintaining the expression of these receptors is important for the characteristics of such progenitor cells. Therefore, it is possible that these molecules are also useful for the identification of progenitor cells derived from primary liver tissues. For in vivo and in vitro proliferation and differentiation, interactions between various cell types through soluble factors are important. This study revealed that paracrine factors such as IGF and TWEAK derived from supporting feeder cells directly regulate the proliferation of human iPS cell-derived HPCs through their specific receptors. A further detailed understanding of the proliferation mechanisms of human iPS cell-derived HPCs is important to establish defined and xeno-free culture methods and will contribute to the realization of regenerative medicine.

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Author Disclosure Statement

No competing financial interests exist.

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