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Identification of Proliferating Human Hepatic Cells From Human Induced Pluripotent Stem Cells

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ABSTRACT

Mass-scale production of hepatocytes from human induced pluripotent stem cells (iPSCs) with functional properties of primary hepatocytes is of great value in clinical transplantation for liver failure as well as in facilitating drug development by predicting humanized drug metabolism profiles. In this report, we generated human hepatocyte-like cells from human iPSCs with the use of a stepwise protocol. Aiming at future clinical and industrial application, it is important to determine the suitable stage of iPSC-derived hepatic cells that possess high proliferative capacity to intensively expand the hepatic cells. Ki67 immunostaining showed that human iPSC-derived hepatic endoderm cells contained Ki67^{+} cells at the highest level in the middle stage of hepatic differentiation, suggesting that the abundance of proliferating hepatic progenitor cells exists in this stage. Extensive expansion and differentiation of human iPSC-derived hepatic progenitors will provide future perspectives in transplantation therapy and drug development.

HUMAN hepatocyte transplantation restores damaged liver function, especially in metabolic diseases, and is considered to be a promising way to replace auxiliary liver transplantation for the treatment of liver failure [1]. However, sources of donor human hepatocytes are lacking. Alternatively, human induced pluripotent stem cells (iPSCs) can now serve as an inexhaustible cell source for hepatocyte transplantation owing to their infinite proliferative capacity and pluripotency to differentiate into a variety type of cells, including hepatocytes [2–5].

During early liver organogenesis or regeneration, hepatic progenitor cells serve as a proliferative pool to derive a number of liver cells in vivo [1]. Hepatic progenitor cells are known to represent a bipotential precursor population, which can simultaneously coexpress epithelial markers typical for cholangiocytes and hepatocytes [6]. Collectively, owing to their robust expanding potential, the generation, expansion, and transplantation of iPSC-derived hepatic progenitors may be therapeutically useful for efficient reconstitution of human liver to treat a variety of liver disorders [1,7,8]. Herein, we generated mature hepatocyte-like cells under a conventional 4-step differentiation protocol. Mature hepatocyte-like cells are successfully generated after a total of 22 days' differentiation under treatment with sequential addition of inductive factors. Furthermore, cell proliferative capability at various differentiation stages was assessed by Ki67 expression level, peaking at the hepatic endoderm stage. These results imply that hepatic endoderm cells might contain abundant hepatic progenitor cells with vigorous expansion potential.

MATERIALS AND METHODS

Culture of Human iPSCs

Human iPSCs were cultured on mitomycin C–treated mouse embryonic fibroblast feeder cells on gelatin (0.1%) (Sigma, St Louis, Missouri)–coated petri dishes in the human iPSC culture medium of DMEM/F12 (1:1; Gibco, New York) supplemented with 25%

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KSR (Gibco), 2 mmol/L Glutamax Supplement (Gibco), 1× MEM nonessential amino acid (Gibco), 0.1 mmol/L 2-mercaptoethanol, and 5 ug/mL human basic fibroblast growth factor (FGF2; R&D Systems, Minnesota).

Differentiation of Human iPSCs into Hepatocyte-like Cells

Procedures for differentiation of human hepatocyte-like cells from human iPSCs have been previous reported [4].

Immunocytochemistry

Cells fixed with 4% paraformaldehyde for 10 minutes were permeabilized with 0.2% Triton X100 in phosphate-buffered saline solution for 5 minutes and stained overnight with anti-Ki67 antibody (Dako, Glostrup, Denmark), anti-FOXA2 (Millipore), anti-SOX17 (R&D Systems), anti-HNF4α (Santa Cruz), and anti-AFP (Mybiosource), and secondary antibodies used were Alexa Fluor 488–conjugated goat antimouse and Alexa Fluor 555–conjugated goat antimouse (Invitrogen). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI).

RESULTS

Differentiation of Human iPSCs toward Hepatocytes

We used a 4-step strategy based on activin A, BMP4/FGF2, HGF, OSM, and Dex for the induction of human iPSC into mature hepatocyte-like cells, which mimic the embryonic development of the liver (Fig 1A). We routinely confirmed the successful maintenance of human iPSCs by checking their morphology and gene expressions. On hepatic differentiation, iPSCs dissociated with Accutase were seeded on Matrigel (1:30 diluted by pure DMEM/F12)–coated dishes at a density of 0.75×10^5 cells/cm^2. After exposed to activin A for 6 days, the majority of cells contained large nuclei and dark granular deposits within nuclei, defined as definitive endoderm cells. Next, cells were cultured for another 3 days in the medium with bone morphogenetic protein 4 (BMP4) and FGF2, and their morphology changed into a small round shape with high nuclear to cytoplasmic ratio, which resembled hepatic endoderm cells. Then, cells were further incubated with hepatocyte growth factor (HGF) for up to 4 days and subsequently with

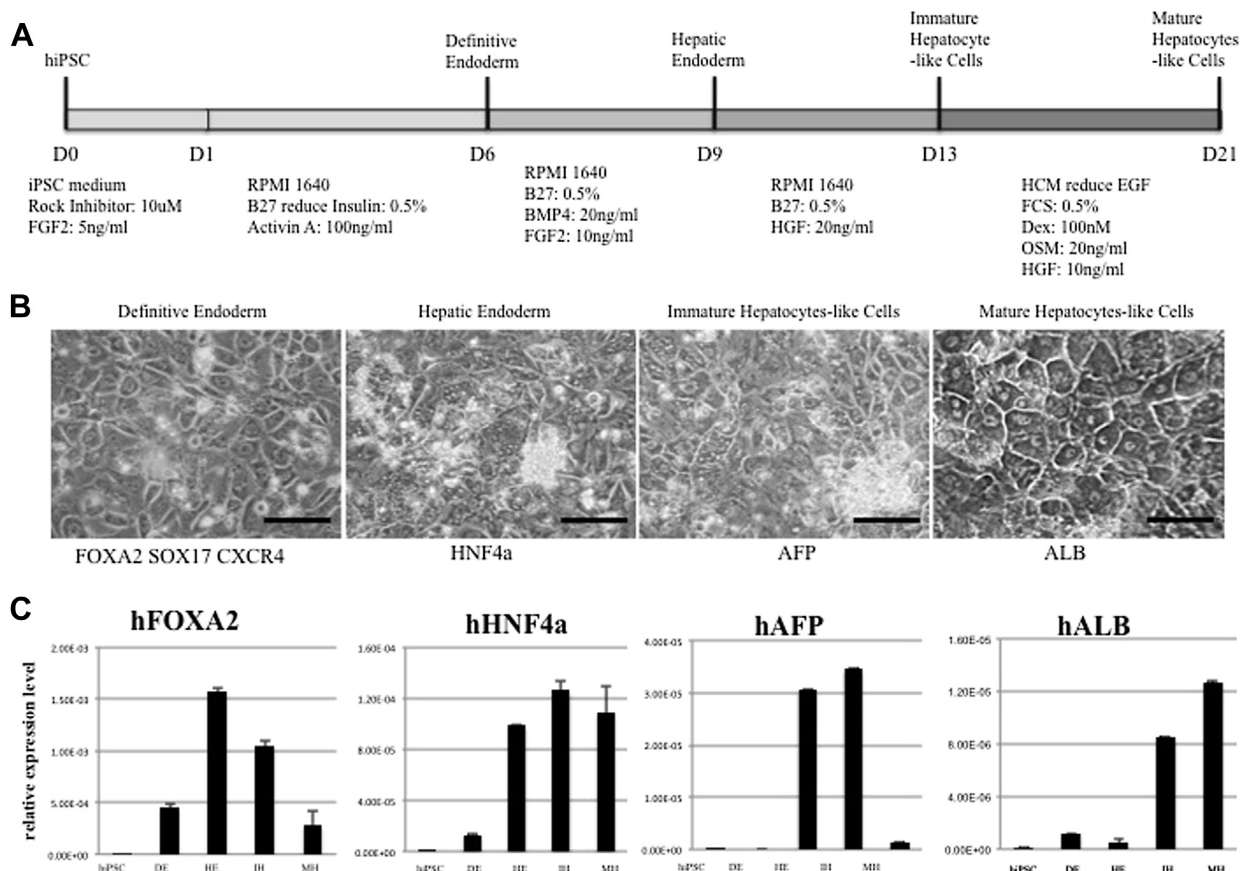

Fig 1. Differentiation of human induced pluripotent stem cells (iPSCs) into hepatocyte-like cells. **(A)** Differentiation timeline detailing the developmental stages (days 0–21) with basal medium and growth factor combinations and concentrations are provided. **(B)** Bright-field pictures showing the differentiation of human iPSCs into hepatocytes according to a natural path of development. Definitive endoderm specification started with activin A treatment and showed an increase in cell size. Hepatic endoderm structures occurred after incubation with BMP4/FGF2 with a dark cytoplasm. Immature hepatocyte-like cells were generated under HGF treatment. Mature hepatocyte-like cells generated after 22 days of differentiation. Scale bars, 100 μm. **(C)** Gene expression of endoderm marker FOXA2 and hepatocyte markers HNF4α, AFP, ALB in different cell stages ($n = 3$ biologic triplicate replicates).

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