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Isolation and expansion of human pluripotent stem cell-derived hepatic progenitor cells by growth factor defined serum-free culture conditions

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ABSTRACT

Limited growth potential, narrow ranges of sources, and difference in variability and functions from batch to batch of primary hepatocytes cause a problem for predicting drug-induced hepatotoxicity during drug development. Human pluripotent stem cell (hPSC)-derived hepatocyte-like cells *in vitro* are expected as a tool for predicting drug-induced hepatotoxicity. Several studies have already reported efficient methods for differentiating hPSCs into hepatocyte-like cells, however its differentiation process is time-consuming, labor-intensive, cost-intensive, and unstable. In order to solve this problem, expansion culture for hPSC-derived hepatic progenitor cells, including hepatic stem cells and hepatoblasts which can self-renewal and differentiate into hepatocytes should be valuable as a source of hepatocytes. However, the mechanisms of the expansion of hPSC-derived hepatic progenitor cells are not yet fully understood. In this study, to isolate hPSC-derived hepatic progenitor cells, we tried to develop serum-free growth factor defined culture conditions using defined components. Our culture conditions were able to isolate and grow hPSC-derived hepatic progenitor cells which could differentiate into hepatocyte-like cells through hepatoblast-like cells. We have confirmed that the hepatocyte-like cells prepared by our methods were able to increase gene expression of cytochrome P450 enzymes upon encountering rifampicin, phenobarbital, or omeprazole. The isolation and expansion of hPSC-derived hepatic progenitor cells in defined culture conditions should have advantages in terms of detecting accurate effects of exogenous factors on hepatic lineage differentiation, understanding mechanisms underlying self-renewal ability of hepatic progenitor cells, and stably supplying functional hepatic cells.

1. Introduction

Primary hepatocytes are used as a tool for predicting drug-induced hepatotoxicity during drug development. However, their limited growth potential, narrow ranges of sources, and difference in variability and functions from batch to batch cause a problem. Generation of homogenous hepatocyte-like cells derived from human pluripotent stem cell (hPSC) is expected as a solution. Several studies have already reported efficient methods that differentiation of hPSCs into hepatocyte-like cells which exhibit hepatic gene expression and functions [1–

Abbreviations: hPSC, human pluripotent stem cell; HPC, hepatic progenitor cell; FGF, fibroblast growth factor; BMP, bone morphogenetic protein; DE, definitive endoderm; hPSC-HPC, hPSC-derived HPC; HPC-HBC, HPC-derived hepatoblast-like cell; HPC-HC, HPC-derived hepatocyte-like cell; hESC, human embryonic stem cell; iPSC, induced pluripotent stem cell; MEF, mouse embryo fibroblast; FAF-BSA, fatty acid–free bovine serum albumin; FOXA2, forkhead box A2; hPSC-HLPC, hPSC-derived hepatic lineage progenitor cell; HNF1α, hepatocyte nuclear factor 1 alpha; HGF, hepatocyte growth factor; BIO, (2′Z,3′E)–6-Bromoindirubin-3′-oxime; EGF, epidermal growth factor; NIC, nicotinamide; DEX, dexamethasone; DAPT, N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester; BDM, biliary differentiation medium; ICG, indocyanine green; HNF4α, hepatocyte nuclear factor 4 alpha; AFP, alpha fetoprotein; GFX, GF109203X; ALB, albumin; CYP3A7, cytochrome P450 3A7; ITGB4, Integrinβ4

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5]. Protocols by Takayama et al. [6,7] employ transduction of adenoviral vector with transcription factor genes. Since adenovirus vectors are one of the most efficient gene delivery vehicles that can provide high transduction efficiency in both dividing and non-dividing cells, differentiation into hepatocyte-like cells should be comparatively stable. Nevertheless, differentiation efficiency into hepatocyte-like cells varies between experiments. Moreover, the differentiation processes toward hepatocyte-like cells from hPSCs are time-consuming, labor-intensive, and cost-intensive, as well as generated hepatocyte-like cells are inhomogeneous. Thus, isolation and expansion of hepatic progenitor cells (HPCs) that have both ability of self-renewal and differentiation into functional hepatocytes would be ideal.

Reid's group [8–10] has proposed that there are 2 types of HPCs which can give rise to liver: hepatic stem cells (HSCs) and hepatoblasts in fetal and adult liver. Recent accumulated studies have demonstrated that HSCs can self-renew and differentiate into hepatoblasts which can differentiate into hepatocytes or bile ductal cells [9,11,12]. During embryonic development, several kinds of hepatic lineage progenitor cells (HLPCs) are generated from ventral foregut endoderm [13]. The ventral foregut endoderm stimulated by fibroblast growth factor (FGF) and bone morphogenetic protein (BMP) gives rise to hepatic diverticulum. The hepatic diverticulum cells expand to form liver bud and differentiate into hepatoblast, which differentiate into hepatocytes or bile ductal cells. Usually, methods that promote hPSCs differentiation into hepatocyte-like cells *in vitro* are comprised of 3 steps: differentiation of hPSCs into definitive endoderm (DE), induction into hepatic lineage, and maturation into hepatocyte-like cells. These findings imply that several types of HLPC, including hepatic stem cells and hepatoblasts which can self-renewal and differentiate into hepatocytes, may exist and have a variety of property in different conditions. Previous studies [14,15] reported that hPSC-derived hepatoblast-like cells can be maintained and expanded under serum containing culture conditions. In current study, we developed growth factor defined serum-free culture conditions for growing hPSC-derived HPCs (hPSC-HPCs), and expanding HPC-derived hepatoblast-like cells (HPC-HBCs) which can differentiate toward hepatocyte-like cells (HPC-HCs). Isolation of hPSC-HPCs in our culture conditions should have advantages to detecting effects of exogenous factors on hepatic lineage differentiation, understanding underlying self-renewal ability of HPCs, and establishing stable supply of functional hepatic cells for pharmaceutical research.

2. Materials and methods

2.1. hPSCs culture

A human embryonic stem cell (hESC) line H9 (WA09) [16,17] was obtained from WISC Bank (WiCell Research Institute, Madison, WI, http://www.wicell.org/). Human lung fibroblast cell MRC-5 [18] -derived induced pluripotent stem cell (iPSC) lines, Tic (JCRB 1331), and Dotcom (JCRB 1327) [19,20] were obtained from the JCRB Cell Bank (National Institutes of Biomedical Innovation, Health and Nutrition, Osaka, Japan, http://cellbank.nibiohn.go.jp/). H9, Tic, and Dotcom cell lines were maintained on irradiated-inactivated mouse embryo fibroblast (MEF) feeder cells in KnockOut™ serum replacement (KSR, Thermo Fisher Scientific, Waltham, MS, https://www.thermofisher.com/)-based medium supplemented with 10 ng/ml FGF2 (Katayama Chemical Industries, Osaka, Japan, http://katayamakagaku.co.jp/) [16]. KSR-based medium consisted of KnockOut™ DMEM/F-12 (Thermo Fisher Scientific) supplemented with 20% KSR, 0.1 mM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com), 2 mM L-glutamine (Thermo Fisher Scientific), 0.1 mM non-essential amino acids (Thermo Fisher Scientific), and 4 or 10 ng/ml human recombinant FGF2. Prior to cell differentiation, H9 and Tic cells were maintained without feeder cells on 2 μg/cm^2 bovine fibronectin (Sigma-Aldrich) in hESF-FX (WO2012/104936) modified from hESF9 medium, which we previously developed for culturing hESCs [21]. hESF-FX medium consisted of mESF basal medium (Wako Pure Chemical Industries, Osaka, Japan, http://www.wako-chem.co.jp/) supplemented with 5 factors (10 μg/ml human recombinant insulin, 5 μg/ml human apo-transferrin, 10 μM 2-ethanolamine, 10 μM 2-mercaptoethanol, 20 nM sodium selenite), 9.4 μg/ml oleic acid conjugated with 1 mg/ml recombinant human serum albumin (all from Sigma-Aldrich), 0.1 mg/ml L-ascorbic acid phosphate (Wako Pure Chemical Industries), 4 or 10 ng/ml FGF2, and 2 ng/ml activin A (R & D Systems, Minneapolis, MN, http://www.rndsystems.com). The experiments using hESCs were performed following the Guidelines for utilization of hESCs of the Ministry of Education, Culture, Sports, Science and Technology of Japan with the approval by the institutional research ethics committee.

2.2. Culture of primary human hepatocytes

Cryopreserved primary human hepatocytes (VERITAS, Tokyo, Japan, http://www.veritastk.co.jp/) were cultured according to the manufacturer's instructions. Briefly, the hepatocytes were seeded at 1.25×10^5 cells/cm^2 in hepatocyte culture medium (Lonza, Basel, Switzerland, http://www.lonza.com/) containing 10% FBS (Thermo Fisher Scientific) onto type I collagen (Nitta gelatin, Sendai, Japan, http://www.nitta-gelatin.co.jp/)-coated plates. The medium was replaced 6 h after seeding. Experiments for drug response were conducted with the hepatocytes that were cultured for 48 h after plating the cells, as previously described [6,7]. Cultures of primary human fetal hepatocytes, human normal fetal liver-CD34+ cells, and HepaRG® cells which are terminally differentiated hepatic cells derived from a human hepatic progenitor cell line are described in Supplementary information.

2.3. Preparation of hPSC-derived hepatic lineage progenitor cells

Hepatic lineage differentiation of Dotcom cells using adenovirus vectors was performed as described previously [6]. Briefly, to promote mesendoderm differentiation, hPSCs were cultured for 2 days on Matrigel (Corning, Corning, NY, https://www.corning.com/) in a differentiation medium consisting of hESF-DIF medium (Cell Science & Technology Institute, Sendai, Japan, http://cstimedia.com/) supplemented with 5 factors, 0.5 mg/ml fatty acid–free bovine serum albumin (FAF-BSA; Sigma-Aldrich), and 100 ng/ml activin A. To generate DE-like cells, hPSC-derived mesendoderm cells were transduced with forkhead box A2 (FOXA2)-expressing adenovirus vectors on day 2 and cultured until day 6 on Matrigel in the differentiation medium. To generate hPSC-HLPCs, the DE-like cells were transduced with FOXA2- and hepatocyte nuclear factor 1α (HNF1α)-expressing adenovirus vectors on day 6 and cultured for 3 days on Matrigel in hepatocyte culture medium (Lonza) supplemented with 30 ng/ml bone morphogenetic protein 4 (BMP4; R & D Systems) and 20 ng/ml FGF4 (R & D Systems). Hepatic lineage differentiation of Tic and H9 cells was performed according to respective methods as described in Supplementary Information.

2.4. Development of culture conditions for hPSC-HPCs and HPC-HBCs

hPSC-HLPCs were seeded on plastic dish coated with bovine fibronectin (Sigma-Aldrich) at 2 μg/cm^2 in a serum-free medium for culturing HPCs, designated as HepSCF medium (Supplementary Table S1). HepSCF medium (JP2015-006137A) consists of HepSCF basal medium supplemented with 5 factors (10 μg/ml human recombinant insulin, 5 μg/ml human apo-transferrin, 10 μM 2-ethanolamine, 10 μM 2-mercaptoethanol, 25 nM sodium selenite), 100 ng/ml bovine heparan sulfate sodium salt (Sigma-Aldrich), and 4 mg/ml FAF-BSA (Merck