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Enrichment of high-functioning human iPS cell-derived hepatocyte-like cells for pharmaceutical research

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ABSTRACT

Human iPS cell-derived hepatocyte-like cells are expected to be utilized in pharmaceutical research. However, the purity of high-functioning hepatocyte-like cells is not high enough. In particular, the purity of cytochrome P450 3A4 (CYP3A4), which is a representative hepatic drug-metabolizing enzyme, positive cells is still quite low (approximately 20%). To address this problem, we established the CYP3A4-NeoR-EGFP transgenic reporter human iPS cell line (CYP3A4-NeoR-EGFP iPS cells) by using genome editing technology. The CYP3A4-NeoR-EGFP iPS cells were differentiated into hepatocyte-like cells, and then the hepatocyte-like cells were treated with neomycin to concentrate the hepatocyte-like cells which strongly express CYP3A4. After the neomycin treatment, the percentage of CYP3A4-positive cells was higher than 80%. The gene expression levels of various drug-metabolizing enzymes, transporters, and hepatic transcription factors were significantly enhanced by neomycin treatment. In addition, the CYP1A2, 2C19, 2D6, and 3A4 activities and biliary excretion capacities were significantly increased by neomycin treatment. We also confirmed that the detection sensitivity of drug-inducing hepatotoxicity was enhanced by neomycin treatment. We succeeded in obtaining human iPS cell-derived hepatocyte-like cells that highly express CYP3A4 at high purity. We believe that our high-purity and high-functioning hepatocyte-like cells could be used to evaluate the risk of drug candidates.

1. Introduction

Human induced pluripotent stem (iPS) cell-derived hepatocyte-like cells are expected to be utilized in pharmaceutical development, such as drug screening and drug metabolism testing. Many research groups, including ours, have been engaged in developing an efficient hepatocyte differentiation protocol from human iPS cells [1–5]. Hepatocyte-like cells have the capacity to secrete albumin (ALB), urea, and bile acid and also have drug metabolism capacity. However, several issues must be overcome before hepatocyte-like cells can be used in pharmaceutical applications. It has been reported that the hepatocyte-like cells are similar to fetal hepatocytes rather than adult hepatocytes [6,7]. In addition, although almost all of the hepatocyte-like cells are positive for early hepatic markers (such as ALB), some of the hepatocyte-like cells are negative for late hepatic markers (such as cytochrome P450 (CYP)) [8]. These facts suggest that it is still difficult to generate mature hepatocyte-like cells at high purity.

To obtain mature hepatocyte-like cells at high purity, some groups have sorted mature hepatocyte-like cells by using hepatocyte-specific cell surface markers [8,9]. Basma et al. have reported that mature hepatocyte-like cells could be concentrated by sorting asialoglycoprotein receptor 1 (ASGR1)-positive cells [9].

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Mallanna et al. have also reported that mature hepatocyte-like cells could be concentrated by sorting sodium taurocholate cotransporting polypeptide (NTCP)-positive cells [8]. However, although cell suspensions of mature hepatocyte-like cells can be obtained by cell sorting, it is difficult to replace the sorted mature hepatocyte-like cells, because hepatocyte-like cells easily lose their hepatic functions during the cell reattachment process. In the drug screening and CYP induction experiments, attached hepatocytes, not suspended hepatocytes, are highly required. Thus, the cell sorting technology might not be suitable for preparing attached mature hepatocyte-like cells at high purity. Therefore, we decided to purify mature hepatocyte-like cells by drug-selection.

In this study, we attempted to establish a CYP3A4-neomycin resistance gene (NeoR)-enhanced green fluorescent protein (EGFP) transgenic reporter human iPS cell line (CYP3A4-NeoR-EGFP iPS cells) by using clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein-9 (Cas9) technology. Although it is known that the genome editing efficiency of human ES/iPS cell clones is quite low at the heterochromatin locus [10,11], we have recently found that RAD51 recombinase (RAD51) overexpression and valproic acid treatment enhanced genome targeting efficiency in human ES/iPS cells regardless of the transcriptional activity of the targeted locus [12]. Therefore, we targeted the *CYP3A4* locus by using our genome editing technology. After the hepatocyte differentiation, we tried to concentrate the hepatocyte-like cells that strongly expressed CYP3A4 by treating the CYP3A4-NeoR-EGFP iPS cell-derived hepatocyte-like cells with neomycin. In addition, we examined the pharmaceutical value of CYP3A4-positive high-functioning hepatocyte-like cells.

2. Materials and methods

2.1. Human iPS cells

The human iPS cell line, Tic [13,14] (provided by Dr. Akihiro Umezawa at the National Center for Child Health and Development), was maintained on 1 μg/cm^2 recombinant human laminin 511 E8 fragments (iMatrix-511, Nippi) with StemFit AK02N medium (Ajinomoto) [15]. Human iPS cell lines, YOW-iPS cells and FCL-iPS cells [16], generated from primary human hepatocytes was maintained on 1 μg/cm^2 iMatrix-511 with StemFit AK02N medium. To passage human iPS cells, near-confluent human iPS cell colonies were treated with TrypLE Select Enzyme (Thermo Fisher Scientific) for 3 min at 37 °C. After the centrifugation, human iPS cells were seeded at an appropriate cell density (5×10^4 cells/cm^2) onto iMatrix-511. Human iPS cells on iMatrix-511 were subcultured every 6 days.

2.2. Hepatocyte differentiation

Before the initiation of hepatocyte differentiation, human iPS cells were dissociated into single cells by using TrypLE Select Enzyme and plated onto a mixture of 0.25 μg/cm^2 laminin 511 E8 fragments (LN511-E8) and 0.75 μg/cm^2 laminin 111 E8 fragments (LN111-E8). Recombinant laminin E8 fragments were produced using a FreeStyle 293 Expression System (Thermo Fisher Scientific) and purified from conditioned media. These cells were cultured in StemFit AK02N medium for 4–6 days. The differentiation protocol for the induction of definitive endoderm cells, hepatoblast-like cells, and hepatocyte-like cells was based on our previous reports with some modifications [16,17]. Briefly, in the definitive endoderm differentiation, human iPS cells were cultured with the RPMI1640 medium (Sigma) containing 100 ng/ml activin A (R&D Systems), 1% GlutaMAX (Thermo Fisher Scientific), and 1 × B27 Supplement Minus Vitamin A (Thermo Fisher Scientific) for 4 days. For the induction of hepatoblast-like cells, the definitive endoderm cells were cultured with RPMI1640 medium containing 20 ng/mL bone morphogenetic protein 4 (BMP4) (R&D Systems) and 20 ng/mL fibroblast growth factor 4 (FGF4) (R&D Systems), 1% GlutaMAX, and 1 × B27 Supplement Minus Vitamin A for 5 days. To perform the hepatocyte differentiation, the hepatoblast-like cells were cultured for 1 day in RPMI1640 medium (Sigma) containing 20 ng/ml HGF, 1% GlutaMAX, 1 × B27 Supplement Minus Vitamin A, and 60 μg/ml type IV collagen (Nitta Gelatin). The cells were then cultured for 4 days in RPMI1640 medium (Sigma) containing 20 ng/ml HGF, 1% GlutaMAX, and 1 × B27 Supplement Minus Vitamin A. Finally, the cells were cultured for 11 days in Hepatocyte Culture Medium (HCM, Lonza) without EGF but with 20 ng/ml oncostatin M (OsM).

2.3. Real-time RT-PCR

Total RNA was isolated from human iPS cells and their derivatives using ISOGENE (NIPPON GENE). cDNA was synthesized using 500 ng of total RNA with a Superscript VILO cDNA synthesis kit (Thermo Fisher Scientific). Real-time RT-PCR was performed with SYBR Green PCR Master Mix (Applied Biosystems) using a StepOnePlus real-time PCR system (Applied Biosystems). The relative quantitation of target mRNA levels was performed by using the $2^{-\Delta\Delta CT}$ method. The values were normalized by those of the housekeeping gene, *peptidylprolyl isomerase A* (*PPIA*). No significant differences were found in the *PPIA* expression levels on the different cDNAs. PCR primer sequences were obtained from PrimerBank (https://pga.mgh.harvard.edu/primerbank/).

2.4. Electroporation

The *CYP3A4* locus was targeted using the donor plasmids and CRISPR/Cas9 plasmids. We combined a NeoR-EGFP cassette with the CYP3A4 gene in the last coding exon (CYP3A4-NeoR-EGFP). Note that the CYP3A4 activities were not changed by combining the NeoR-EGFP cassette with CYP3A4 (Fig. S1). The efficient targeting experiments of human iPS cells were performed as we previously described [12]. Briefly, human iPS cells were treated with 10 μM valproic acid (VPA) for 24 h. Human iPS cells (1.0×10^6 cells) were dissociated into single cells by using TrypLE Select Enzyme, and resuspended in prewarmed Nucleofector Solution (Lonza). The electroporation was performed by using a 4D-Nucleofector System and 4D-Nucleofector Kit (P3) (both from Lonza) according to the manufacturer's instructions. The ratio of Nucleofector Solution to the plasmid solution was 90 μL: 10 μL (total 100 μL). The plasmid solution consists of 4 μg donor plasmids, 4 μg CRISPR/Cas9 plasmids (two CRISPR/Cas9 vectors carrying different sgRNA were mixed), 1 μg RAD51-expressing plasmids, and 1 μg puromycin resistant protein (PuroR)-expressing plasmids. PuroR-expressing plasmids were transfected to select the cells which were correctly transfected. After the electroporation, the cells were seeded onto 1 μg/cm^2 iMatrix-511-coated dishes and cultured with StemFit AK02N medium containing 10 μM ROCK inhibitor. After culturing for 2 days, the medium was replaced with 10 μM puromycin-containing medium. Then, 48 h after its addition, the puromycin-containing medium was removed and the original medium was added. At 10 days after the electroporation, 24 individual colonies were picked up, and then seeded onto a 1 μg/cm^2 iMatrix-511-coated 24-well plate. After most of the wells became nearly confluent, PCR and sequencing analysis were performed to examine whether the clones were correctly targeted.

2.5. ELISA

The culture supernatants, which were incubated for 24 h after