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Differentiation of Human Induced Pluripotent Stem Cells into Functional Enterocyte-like Cells Using a Simple Method

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Summary: Human induced pluripotent stem (iPS) cells were differentiated into the endoderm using activin A and were then treated with fibroblast growth factor 2 (FGF2) for differentiation into intestinal stem cell-like cells. These immature cells were then differentiated into enterocyte-like cells using epidermal growth factor (EGF) in 2% fetal bovine serum (FBS). At the early stage of differentiation, mRNA expression of caudal type homeobox 2 (CDX2), a major transcription factor related to intestinal development and differentiation, and leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5), an intestinal stem cell marker, was markedly increased by treatment with FGF2. When cells were cultured in medium containing EGF and a low concentration of FBS, mRNAs of specific markers of intestinal epithelial cells, including sucrase–isomaltase, the intestinal oligopeptide transporter SLC15A1/peptide transporter 1 (PEPT1), and the major metabolizing enzyme CYP3A4, were expressed. In addition, sucrase–isomaltase protein expression and uptake of β-Ala-Lys-N-7-amino-4-methylcoumarin-3-acetic acid (β-Ala-Lys-AMCA), a fluorescence-labeled substrate of the oligopeptide transporter, were detected. These results demonstrate a simple and direct method for differentiating human iPS cells into functional enterocyte-like cells.

Keywords: human iPS cells; intestinal differentiation; enterocytes; pharmacokinetics; drug metabolizing enzymes; drug transporters

Introduction

The small intestine and liver play important roles in all aspects of pharmacokinetics, including drug disposition, drug metabolism, drug transport, drug interactions, and bioavailability. Because drug-metabolizing enzymes such as cytochrome P450 (CYP) and UDP-glucuronyltransferase (UGT) and drug transporters such as ATP-binding cassette (ABC) and solute carrier (SLC) transporters are appreciably expressed in the small intestinal epithelia,[1,2)] it is necessary to estimate intestinal metabolism and absorption during the early stages of drug development. To this end, various *in vivo* and *in vitro* systems have been employed to assess the intestinal first-pass effect. However, extrapolation of experimental animal data to humans is often hampered by species differences, and primary human intestinal cells are rarely available. Therefore, a system that accurately and easily estimates intestinal membrane permeability and metabolism is urgently required.

Human induced pluripotent stem (iPS) cells can be generated by transducing reprogramming factors (OCT3/4, SOX2, KLF4, c-MYC) into somatic cells[3)] and these cells share many characteristics of embryonic stem (ES) cells.[4)] Human iPS cells are expected to be useful not only in regenerative medicine but also in pharmacokinetic and toxicokinetic drug development studies because their use is not as ethically regulated as that of human ES cells.

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Therefore, human iPS cells have been differentiated into various cell types, including pancreatic cells,[5,6] neuron cells,[7] cardiomyocytes,[8] and hepatocytes.[9–13]

A few studies report the differentiation of iPS cells into enterocytes. In particular, mouse iPS cells were differentiated into a gut-like organ following the formation of embryoid bodies (EBs),[14] and human iPS cells were differentiated into intestinal tissue using a culture method for intestinal crypt stem cells.[15] However, functional characteristics of drug transporters and drug-metabolizing enzymes of differentiated cells are almost entirely unexplored in these reports. Thus, whether differentiated intestinal tissue or organoids can be used in drug development studies, particularly studies of the absorbability and metabolic capacity of drugs, remains unclear.

The small intestinal epithelium comprises absorptive cells, goblet cells, endocrine cells, and Paneth cells. Several signaling pathways such as Notch, Wnt, phosphoinositide 3-kinase, and bone morphogenic protein signaling are associated with intestinal development.[16] Leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5) has been identified as an intestinal stem cell marker.[17] Indeed, this was also observed in mouse LGR5-positive cells that formed a crypt–villus structure *in vitro*.[18] Improvements in this technique have enabled long-term culture of human epithelial cells isolated from the small intestine,[19] leading to advances in intestinal stem cell research. However, mechanisms of intestinal development are not sufficiently understood, and it is difficult to control differentiation into all four cell types.

In this study, we established a functional enterocyte-like cell line from human iPS cells for use in drug development studies. We propose a simple and direct differentiation method by two-dimensional culture. Our data may facilitate the development of an intestinal pharmacokinetic analysis system to identify safe drugs with favorable pharmacokinetic characteristics.

Materials and Methods

Materials: FGF2, FGF4, activin A, and epidermal growth factor (EGF) were purchased from PeproTech Inc. (Rocky Hill, NJ). Wnt3a was purchased from R&D Systems, Inc. (Minneapolis, MN). BD Matrigel matrix Growth Factor Reduced (Matrigel) was purchased from BD Biosciences (Bedford, MA). Affinity-isolated rabbit polyclonal antihuman sucrase–isomaltase antibody and intestinal recombinant protein epitope signature tags were purchased from Sigma-Aldrich Co. (St. Louis, MO). The purified IgG fraction of polyclonal goat antiserum against rabbit IgG conjugated with Alexa Fluor 568 and KnockOut Serum Replacement (KSR) were purchased from Invitrogen Life Technologies Co. (Carlsbad, CA). β-Ala-Lys-N-7-amino-4-methylcoumarin-3-acetic acid (β-Ala-Lys-AMCA) was purchased from BIOTREND Chemicals (Destin, FL), and (+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl)cyclohexanecarboxamide dihydrochloride (Y-27632) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Human adult small intestine total RNA from a 66-year-old male donor was purchased from BioChain Institute Inc. (Newark, CA). Murine embryonic fibroblasts (MEFs) were obtained from Oriental Yeast Co. (Tokyo, Japan). The RNeasy Mini Kit was purchased from Qiagen (Valencia, CA). The PrimeScript RT Reagent Kit and TaKaRa SYBR Premix EX Taq II were purchased from Takara Bio Inc. (Otsu, Japan). All other reagents were of the highest quality available.

Human iPS cell cultures: A human iPS cell line (Windy) was provided by Dr. Akihiro Umezawa of the National Center for Child Health and Development. Human iPS cells were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's nutrient mixture F-12 (DMEM/F12) containing 20% KSR, 2 mM L-glutamine, 1% MEM nonessential amino acid solution (NEAA), 0.1 mM 2-mercaptoethanol, and 5 ng/ml FGF2 at 37°C in humidified air with 5% CO_2. The human iPS cells were cultured on a feeder layer of mitomycin C-treated MEFs, and the medium was changed every day.

Differentiation into enterocyte-like cells: The human iPS cells were used for differentiation studies between passages 30 and 50. When the cells reached approximately 70% confluence, differentiation was initiated by replacing the medium with Rosewell Park Memorial Institute (RPMI) 1640 medium containing 2 mM GlutaMAX, 0.5% fetal bovine serum (FBS), 100 ng/ml activin A (a member of the transforming growth factor-β family that is known to efficiently induce differentiation into the definitive endoderm),[20,21] 100 units/ml penicillin, and 100 µg/ml streptomycin. After 48 h, the medium was replaced with RPMI 1640 containing 2 mM GlutaMAX, 2% FBS, 100 ng/ml activin A, 100 units/ml penicillin, and 100 µg/ml streptomycin, and the cells were cultured for 24 h. Subsequently, the culture medium was replaced with DMEM/F12 containing 2% FBS, 2 mM GlutaMAX, and 250 ng/ml FGF2 or FGF4 with or without 50 ng/ml Wnt3a for 96 h. The cells were then treated for 1 h with the selective Rho-associated kinase inhibitor Y-27632 at 10 µM.[22,23] The cells were then passaged on Matrigel-coated 24-well plates and cultured in DMEM/F12 containing 2% or 10% FBS, 2% B-27 supplement, 1% N2 supplement, 1% NEAA, 2 mM L-glutamine, antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin), and 20 ng/ml EGF for 1, 4, 13, or 19 days. Y-27632 was added at 10 µM during the initial 24 h of culture. The medium was changed every 3 days (**Fig. 1**).

RNA extraction and reverse transcription reaction: Total RNA was isolated from differentiated iPS cells using the RNeasy Mini Kit. First-strand cDNA was prepared from 500 ng of total RNA. The reverse transcription reaction was performed using the PrimeScript RT Reagent Kit according to the manufacturer's instructions.

Real-time polymerase chain reaction (PCR) analysis: Relative mRNA expression levels were determined using SYBR Green real-time quantitative reverse transcription-PCR (RT-PCR). Real-time PCR analysis was performed on the Applied Biosystems 7300 Real Time PCR System using 7300 System SDS software version 1.4 (Applied Biosystems, Carlsbad, CA). PCR was performed with the primer pairs listed in **Table 1** using SYBR Premix EX Taq II. mRNA expression levels were normalized relative to that of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

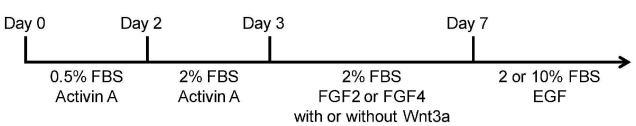

Fig. 1. Schematic of the protocol for the differentiation of human iPS cells into enterocytes
Human iPS cells were cultured in the presence of activin A (100 ng/ml) for 3 days. The cells were further cultured in medium containing FGF2 (250 ng/ml) or FGF4 (250 ng/ml) with or without Wnt3a (50 ng/ml) for 4 days. After 7 days of differentiation, the cells were treated with Y-27632 (10 µM), passaged, and subsequently cultured in the presence of 2% or 10% FBS and EGF (20 ng/ml) for 19 days.