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# Selective Culture Method for Hepatocyte-like Cells Differentiated from Human Induced Pluripotent Stem Cells

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**Summary:** This study aimed to establish culture conditions which are able to give the differentiation of induced pluripotent (iPS) cells to hepatocytes. To this end, we examined the usefulness of a culture medium containing the components involved in the intermediary metabolism in the liver. More specifically, we examined the effect of the "modified L-15 medium" containing galactose, phenylalanine and ornitine, but deprived of glucose, tyrosine, arginine and pyruvic acid. The medium was altered according to changes in the expression of enzymes that participate in liver-specific pathways. After 25 days of differentiation, the differentiated cells expressed hepatocyte markers and drug-metabolizing enzymes. These expression levels were increased using modified L-15 medium. The survival of human fetal liver cells and the death of human fibroblasts were observed during culture in modified L-15 medium. Most of the cells that differentiated from human iPS cells using modified L-15 medium were stained by anti-human albumin antibody. These results suggest that iPS cells can be converted to high purity-differentiated hepatocytes by cultivating them in modified L-15 medium.

**Keywords: induced pluripotent stem cells; differentiation; hepatocytes; selection medium; cytochrome P450; energy sources**

## Introduction

Human induced pluripotent stem (iPS) cells have been generated directly from human fibroblast cells by inducing their expression of defined reprogramming factors (OCT3/4, SOX2, KLF4, and c-MYC).[1)] Recent studies have shown that iPS cells are comparable to embryonic stem (ES) cells because they exhibit the potential for multilineage differentiation and intensive *in vitro* proliferation. The utilization of human iPS cells is anticipated in a variety of applications, including drug development studies involving the prediction of hepatic drug metabolism and liver toxicity. Furthermore, human iPS cell-derived hepatocytes may represent a source for cell transplantation to treat severe liver diseases in the future. Previous studies have already reported hepatocyte differentiation from human iPS cells using cytokines, such as growth factors, transcription factor overexpression by virus vectors, and co-culture with other cells.[2–8)] However, it is difficult to obtain large numbers of highly pure human iPS cell-

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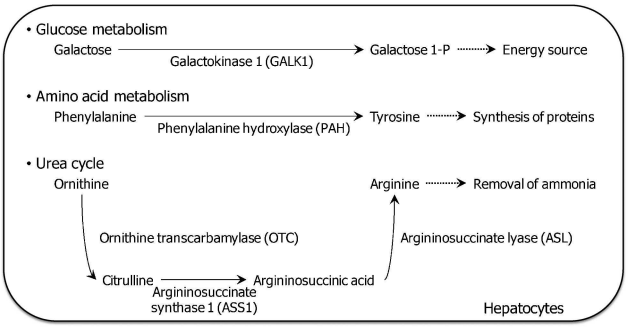


**Fig. 1. Hepatocyte-specific metabolic pathways**
Hepatocytes express galactokinase 1 (GALK1), phenylalanine hydroxylase (PAH), ornithine transcarbamylase (OTC), argininosuccinate synthase 1 (ASS1), and argininosuccinate lyase (ASL). Galactose is metabolized into galactose 1-phosphate by GALK1. Finally, galactose 1-phosphate is transformed into glucose 6-phosphate, and undergoes glycolysis for energy generation. Phenylalanine is metabolized into tyrosine by PAH. Ornithine is transformed into arginine by OTC, ASS1, and ASL. Glucose, tyrosine, and arginine are essential substrates for cell survival. Hepatocytes can utilize substrates by metabolizing galactose, phenylalanine, and ornithine to galactose 1-phosphate, tyrosine, and arginine, respectively, if the essential substrates are not present.

derived hepatocytes both simply and inexpensively using these methods.

Cells have many metabolic pathways and can produce essential nutrients autonomously. Tohyama *et al.* cultured human ES/iPS cell-derived cardiomyocytes in a glucose-depleted culture medium containing abundant lactate and found that only cardiomyocytes survived.[9] Using this approach, human ES/iPS cell-derived cardiomyocytes that did not form tumors after transplantation were obtained with a purity of up to 99%. Hepatocytes also have specific metabolic pathways, which involving glucose and amino acid metabolism, and the urea cycle (**Fig. 1**). Therefore, it is possible that only hepatocytes can survive in a medium that contains the energy sources metabolized by these pathways. Galactokinase 1 (GALK1), which is involved in the conversion of galactose to glucose, is included because glucose is an essential energy source for cells. GALK1 is highly expressed in the liver and kidney.[10,11] Phenylalanine hydroxylase (PAH), which participates in the conversion of phenylalanine to tyrosine, is included because tyrosine is decomposed to acetoacetate and fumarate, which then enters the citric acid cycle and generates energy.[12] Furthermore, tyrosine is used by cells to synthesize proteins and it participates in signal transduction processes such as tyrosine kinase signaling, which functions as an on/off switch for many cellular functions. PAH activity is found in the liver and kidney.[12] Ornithine transcarbamylase (OTC), argininosuccinate synthase 1 (ASS1), and argininosuccinate lyase (ASL), which are involved in the conversion of ornithine to arginine in the urea cycle, must be included because arginine plays an important role in cell division, wound healing, and removal of ammonia, which is toxic to the human body.[13]

It was previously reported that a medium lacking glucose, tyrosine, and arginine, but including galactose, phenylalanine, and ornithine, enriched the hepatoblast-like cells that differentiated from mouse ES cells.[14] A similar medium reportedly eliminated undifferentiated human iPS cells.[15] However, a simple and safe selective culture method for human iPS cell-derived hepatocytes is not available. In the present study, we focused on liver-specific metabolic pathways and attempted to selectively culture hepatocytes differentiated from human iPS cells using a medium containing energy sources metabolized by liver-specific energy metabolic enzymes.

## Materials and Methods

**Materials:** Activin A and hepatocyte growth factor (HGF) were purchased from PeproTech Inc. (Rocky Hill, NJ). Fetal bovine serum (FBS) was purchased from Biowest SAS (Nuaillé, France). Accutase™ was purchased from MS TechnoSystems (Osaka, Japan). Oncostatin M (OSM) and Y-27632 were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). BD Matrigel™ Matrix Growth Factor Reduced (Matrigel™) was purchased from BD Biosciences (Bedford, MA). Mouse monoclonal anti-human albumin (ALB) antibody was purchased from Abcam (Cambridge, UK). KnockOut™ serum replacement (KSR), KnockOut™ Dulbecco's modified Eagle's medium (KO-DMEM), and Alexa Fluor® 568 goat anti-mouse IgG were purchased from Invitrogen Life Technologies Co. (Carlsbad, CA). Human fetal liver total RNA from a 38-week-old male donor and human adult liver total RNA from a 64-year-old male donor were purchased from BioChain Institute Inc. (Newark, CA). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was purchased from Dojindo Laboratories (Kumamoto, Japan). Cosmedium 004 (Cosmedium) was purchased from Cosmo Bio Co., Ltd. (Tokyo, Japan). L-15 medium E, which does not contain glucose, tyrosine, arginine, serum, or pyruvic acid, was provided by Cosmo Bio Co., Ltd. All other reagents were of the highest quality available.

**Cell culture:** Cryopreserved human primary hepatocytes (lot. HPCH10/0910463; 10 donors aged 32–76 years) were obtained from XenoTech, LLC (Lenexa, KS) and thawed using thawing medium without additives (Cat. No. MIL261; Biopredic International, Rennes, France), according to the manufacturer's instructions. Cells were plated on collagen I-coated plates in basal hepatic cell medium (Cat. No. MIL600; Biopredic International) containing additives for hepatocyte seeding medium (Cat. No. ADD221; Biopredic International) for 12 h. The medium was then replaced with basal hepatic cell medium containing additives for hepatocyte culture (Cat. No. ADD222; Biopredic International), and the cells were cultured for 36 h.

The human iPS cell line (Windy), derived from human embryonic lung fibroblast cell line MRC-5, was kindly provided by Umezawa *et al.* of the National Institute for Child Health and Development, Tokyo, Japan. Undifferentiated human iPS cells were cultured based on a previously reported method.[16]

HepG2 cells (Cell No: RCB0523; human hepatocyte carcinoma cells) and HFL-III (Cell No: RCB1886; human fibroblasts derived from the embryonic lung) cells were obtained from the Riken BRC through the National Bio-Resource Project of the MEXT, Japan. These cells were cultured in DMEM containing 10% FBS, 2 mM L-glutamine, and 1% minimal essential medium with non-essential amino acids (MEM NEAA). Human fetal liver (hFL) cells were obtained from the Applied Cell Biology Research Institute (Kirkland, WA) and were cultured in Williams' medium E containing 10% FBS, 2 mM L-glutamine, and 1% MEM NEAA.

**Hepatocyte selection medium:** We produced a hepatocyte selection medium and a control medium based on L-15 medium E. L-15 medium E was produced from Leibovitz's L-15 medium by removing glucose, tyrosine, arginine, serum, and pyruvic acid. Modified L-15 medium, *i.e.*, the hepatocyte selection medium, contained galactose (100, 200, 450, or 900 mg/L) with either FBS