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# Growth and differentiation of colony-forming human hepatocytes in vitro

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***Background/Aims*: Parenchymal hepatocytes (PHs) of rat contain colony-forming parenchymal hepatocytes (CF-PHs) as a small fraction. We aimed to demonstrate the presence of CF-PHs in humans and characterize them with respect to growth and differentiation potential.**

***Methods*: Human PHs were co-cultured with Swiss 3T3 cells in the medium containing human serum, EGF, nicontinamide, and ascorbic acid 2-phosphate. To examine differentiation potential hepatocytes were cultured on gels of Matrigel Matrix.**

***Results*: Few PHs formed colonies, the colony-forming efficiency being as low as 0.01–0.09%. The CF-PHs could be subcultured up to 7 passages. They showed a liver epithelial cell-like morphology, and immunocytochemically positive for albumin (ALB), cytokeratin (CK) 7, 8, 18, and 19 in a pre- and early phase-confluence, whereas they showed a typical differentiated hepatocyte-like morphology, and positive for $\alpha_1$-antitrypsin, but negative for CK7 and 19 in condensed regions at confluence. The CF-PHs at late confluence expressed mRNAs of ALB, HNF4, and isoforms of cytochrome P450 at low levels. However, when cultured on Matrigel, these cells expressed them at high levels comparable to those of original PHs.**

***Conclusions*: We concluded that the human liver contains highly replicative hepatic progenitor-like cells as a minute population that retain a normal differentiation potential.**



***Keywords*: Progenitor cells; Human hepatocytes; Spheroids; Matrigel; Hepatocyte propagation; Albumin; HNF4; Cytochrome P450; Cytokeratins; 3-dimensional culture**

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## 1. Introduction

Generally, hepatocytes in vitro can be maintained as replicating differentiated cells for a limited period. We devised a culture medium, hepatocyte clonal growth medium (HCGM), that supports the growth of rat hepatocytes for a longer period [1–3]. Using HCGM we were able to demonstrate the presence of the colony-forming parenchymal hepatocytes (CF-PHs) as small hepatocytes (SHs) that have a much higher growth potential than conventional PHs [3]. We also showed the presence of replicative human SHs that continue to increase the colony size up to around 35 days when cultured in modified HCGM containing human serum (HS) and Swiss 3T3 cell-conditioned medium (3T3-CM) [4]. However, CF-PHs have not been isolated as a pure fraction and, thus, not been characterized well yet.

The present study was performed, firstly, to determine the occupancy of CF-PHs in the human liver, secondly, to propagate them by serial subculture, and, thirdly, to characterize them in terms of differentiation potential. As a result, we demonstrated the presence of CF-PHs in human PHs and were able to obtain a pure fraction of CF-PHs by serial subcultivation. CF-PHs were characterized in terms of growth potential and differentiation capacity.

## 2. Materials and methods

### 2.1. Isolation, cryopreservation, and thawing of human hepatocytes

The present study was performed under the ethical approval of the Hiroshima Prefectural Institute of Industrial Science and Technology Ethics Board. Liver tissues were obtained from 15 donors (Table 1) in hospitals after receiving their consent before operations according to the 1975 Helsinki declaration.

Hepatocytes were isolated as previously [4] with some modifications. Previously we isolated a SH-containing NPC fraction as the centrifugal supernatant at $50\times g$ for 1 min [4]. In the present study, we isolated PHs as pellets at $50\times g$ for 2 min, because their colony-forming efficiency (0.063%) was much higher than that (0.013%) at $50\times g$ for 1 min. Some of the isolated PHs were cryopreserved and thawed as previously [5]. These cryopreserved and thawed (C–T) PHs were dubbed C–T PHs. The viability of cells was determined by the trypan blue exclusion test. Cells were counted with a hemocytometer. We also used cryopreserved human hepatocytes from a 9-month-old Caucasian boy (IVT079) provided by In Vitro Technologies Inc. (Baltimore, MD). The hepatocytes were thawed as described above. These hepatocytes were called C–T 9MM.

### 2.2. Culture of PHs

PHs were inoculated at $8\times10^3$ cells/cm$^2$ in 35-mm dishes (Becton Dickinson Labware, Franklin, NJ) containing 1.8 ml of h-HCGM for cultures and on Sumilon Celldesks (Sumitomo Bakelite, Tokyo, Japan) in 24-well plates (Becton Dickinson Labware) containing 0.4 ml of h-HCGM for immunocytochemical examinations except that PHs isolated from donor No. 16F1 were inoculated at $4\times10^3$ cells/cm$^2$ in dishes and Celldesks. The ingredients and their concentrations of h-HCGM were identical to the previously reported HCGM [4] except that 3T3-CM and dimethylsulfoxide (DMSO) were not incorporated. The cells were cultured at 37 °C in a 5% $CO_2$-incubator. Swiss 3T3 cells (American Type Culture Collection, Rockville, MD) were treated with mitomycin C [6] and were added at $4\times10^3$ cells /cm$^2$ to the cultures at the next day after the start of culture and every 10 days during culture. h-HCGM was refilled twice per week during culture.

PHs in primary culture grew and became confluent in 35-mm dishes around 30 days after plating. For serial subculturing, the cells were detached by treating with 0.25% trypsin and 1 mM EDTA, and inoculated at $4\times10^3$ cells/cm$^2$ in new 35-mm dishes. Swiss 3T3 cells were incorporated into the dishes as above.

The concentration of albumin (ALB) in culture media was determined with Human ALB ELISA Quantitation Kit (Bethyl laboratories Inc., Montgomery, TX). Cellular proteins were quantified with Protein Assay Kit (Bio-Rad, Hercules, CA) as previously [7].

### 2.3. Determination of colony-forming efficiency of PHs

PHs were cultured on Celldesks, fixed in −30 °C ethanol at 20 days, and stained with anti-cytokeratin (CK) 18 mouse monoclonal antibodies (Amersham Pharmacia Biotech, Piscateway, NJ). Hepatocyte colonies were counted as clusters containing more than 8 CK18-positive (CK18$^+$) cells, and the colony-forming efficiency was calculated by dividing the number of colonies by the number of inoculated PHs.

**Table 1**
**Sources of liver tissues**

| Donor no. | Age | Sex | Disease of excised tissues | Wet weight (g) | Viability (%) |
|---|---|---|---|---|---|
| 3M | 3 | Male | Neuroblastoma | 3.4 | 93.9 |
| 12M | 12 | Male | Hepatocellular carcinoma | 7.9 | 91.5 |
| 16F1 | 16 | Female | Focal nodular hyperplasia | 40.4 | 88.5 |
| 16F2 | 16 | Female | OTC* deficiency | 32.5 | 94.5 |
| 45F | 45 | Female | Metastatic liver tumor | 27.4 | 75.8 |
| 51M | 51 | Male | Metastatic liver tumor | 29.5 | 89.5 |
| 53M | 53 | Male | Metastatic liver tumor | 10.6 | 91.6 |
| 53M2 | 53 | Male | Metastatic liver tumor | 16.4 | 93.8 |
| 58F | 58 | Female | Cholangioma | 6.4 | 95.5 |
| 61F | 61 | Female | Metastatic liver tumor | 4.4 | 80.4 |
| 63F | 63 | Female | Metastatic liver tumor | 15.7 | 89.3 |
| 63M | 63 | Male | Metastatic liver tumor | 5.4 | 87.3 |
| 68F | 68 | Female | Metastatic liver tumor | 27.2 | 92.2 |
| 72M1 | 72 | Male | Metastatic liver tumor | 14.4 | 40.0 |
| 72M2 | 72 | Male | Metastatic liver tumor | 24.2 | 93.5 |

*Ornithine transcarbamylase.