

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

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## Abstract

**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, nicotinamide, 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.

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

## 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 x g for 1 min [4]. In the present study we isolated PHs as the pellets at 50 x g for 2 min, because their colony-forming efficiency (0.063%) was much higher than that (0.013%) at 50 x 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  $8 \times 10^3$  cells/cm<sup>2</sup> 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  $4 \times 10^3$  cells/cm<sup>2</sup> 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<sub>2</sub>-incubator. Swiss 3T3 cells (American Type Culture Collection, Rockville, MD) were treated with mitomycin C [6] and were added at  $4 \times 10^3$  cells/cm<sup>2</sup> 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  $4 \times 10^3$  cells/cm<sup>2</sup> 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,

Piscataway, NJ). Hepatocyte colonies were counted as clusters containing more than 8 CK18-positive (CK18<sup>+</sup>) cells, and the colony-forming efficiency was calculated by dividing the number of colonies by the number of inoculated PHs.

#### 2.4. Immunocytochemistry

Cells were fixed in ethanol at -30°C. The following primary antibodies were used: rabbit antibodies against human ALB (Dakopatts, Glostrup, Denmark), human  $\alpha_1$ -antitrypsin ( $\alpha_1$ -AT, Dakopatts), human  $\alpha_1$ -fetoprotein (AFP, Dakopatts), and mouse monoclonal antibodies against CK8 (Amersham Pharmacia Biotech), CK18, CK7, and CK19 (Progen Biotechnik GmbH, Bretonneux, France). Antibodies were visualized as previously [2].

#### 2.5. Culture of cells in spheroids

Matrigel Matrix solution (BD Biosciences, Bedford, MA) was dissolved in DMEM at 5 mg/ml, 2 ml of which was added into each well of 6-well plates. The plates were incubated at 37°C for 30 min. CF-PHs from donor No. 12M (12M-CF-PHs) at 3 passages in subculture or HepG2 cells were inoculated at 10<sup>5</sup> cells/cm<sup>2</sup> onto the gels, and were cultured at 37°C (“spheroid culture”) in h-HCGM. At 24 h after plating the media were changed to d-HCGM prepared by adding DMSO at 1% to h-HCGM, and removing nicotinamide and HS from it. The d-HCGM was refilled every 3 days during culture. Spheroids were recovered with BD Cell Recovery Solution (BD Biosciences).

#### 2.6. Quantification of mRNAs

We examined whether C-T procedures had caused breaks in RNA of PHs. Total RNAs were extracted from fresh and C-T PHs of 3 donors, 51M, 53M2, and 68F, and electrophoresed on agarose gels. There were no significant differences in the banding patterns of 28S and 18S RNA between fresh and the corresponding C-T PHs, which indicated that the C-T procedure did not cause such breaks.

12M-CF-PHs at 3 passages in subculture were divided into two groups. One group was cultured in dishes as monolayers and the other group was cultured on Matrigel as

spheroids. HepG2 cells were similarly treated. Total RNAs were obtained from these 4 different cell preparations. cDNAs were synthesized using 1 µg of total RNA, PowerScript RT (Clontech laboratories, Inc., Palo Alto, CA) and Oligo(dT)12-18 primers according to the manufacturer's instruction. mRNAs of genes indicated in the text were measured by real-time quantitative RT-PCR using the previous primers [5] except those for AFP (forward, 5'-CAGCCAAAGTGAAGAGGGAAGA; reverse, 5'-CAGCTTGTGACAGGTTCTGGA) and a SYBR Green dye [8]. Copy numbers of mRNA in the cDNA samples were calculated as previously [8].

### **3. Results**

#### *3.1. Growth and morphology*

C-T 9MM-PHs were thawed, seeded on dishes, and co-cultured with Swiss 3T3 cells up to 20 days (Fig. 1A-H). Some of the attached hepatocytes were marked and monitored for their growth during the culture. A small number of single cells started to divide at 5 days, grew slowly but steadily, and formed colonies. Similarly, freshly isolated 16F2-PHs were cultured up to 33 days for the observation of cell morphology (Fig. 1I and J). The cells showed a liver epithelial cell-like (flat and spread) morphology at 20 days when they were in a growing phase (Fig. 1I). They grew to confluence at 33 days after plating (Fig. 1J). The cells in condensed regions showed a typical hepatocyte-like (cuboidal and densely packed) morphology. PHs were obtained from 11 donors whose age ranged from 3 to 72 years and cultured for 20 days. The colony-forming efficiency was variable from 0.01% to 0.09%, but the efficiency significantly decreased as donors aged (Fig. 2A).

#### *3.2. Growth of CF-PHs in subculture*

PHs from both younger donors, 3M, 12M, 16F2, and older ones, 63M and 72M1, grew under serial subculture (Fig. 2B). Population doubling time (PDT) at passage 4 of older hepatocytes were larger than that of younger ones. The possible maximum passage number of CF-PHs of younger and older donors was 7 and 5, respectively. These results

indicated that the growth potential of CF-PHs is correlated with the age of donor.

### 3.3. Phenotypes of CF-PHs in subculture

The expression of liver-specific marker proteins was determined during subculture of 3M- and 12M-CF-PHs. There were no noticeable differences in their expression profiles between 3M- and 12M-CF-PHs. 3M-CF-PHs at 2 passages were cultured up to 13 days when they were still in a pre-confluence or an early phase of confluence. These cells were CK8<sup>+</sup> (Fig. 3A) and CK18<sup>+</sup> (data not shown), markers of both hepatocytes and bile duct cells, and CK7<sup>+</sup> (data not shown) and CK19<sup>+</sup> (Fig. 3B), markers of bile duct cells. 12M-CF-PHs at 3 passages were further cultured up to 27 days when they became confluent. They were ALB<sup>+</sup> (Fig. 3C), a universal hepatocytic marker. Especially, CF-PHs in condensed regions were heavily ALB<sup>+</sup>. CF-PHs expressed  $\alpha_1$ AT, a marker of mature hepatocytes, in condensed regions, but not in non-condensed regions (Fig. 3D). These cells were AFP<sup>+</sup> (Fig. 3E), a marker of immature or neoplastic hepatocytes. The CF-PHs were also subjected to double immuno-staining for  $\alpha_1$ -AT and CK19 (Fig. 3G, H, and I). CF-PHs in condensed regions were strongly  $\alpha_1$ AT<sup>+</sup> (Fig. 3G and I), but CK19<sup>-</sup> (Fig. 3H and I). Double immuno-staining of  $\alpha_1$ AT and CK7 showed that the CK7 expression pattern was identical to that of CK19 (data not shown).

### 3.4. Phenotypes of CF-PHs in spheroids

12M-CF-PHs at 3 passages were detached from dishes, and inoculated in dishes (Fig. 4A) or on Matrigel (Fig. 4C), and cultured for 7 days. Similarly, HepG2 cells were cultured as a reference cell (Fig. 4B and D). Both types of cells became confluent at 7 days in dishes (Fig. 4A and B). On Matrigel, both 12M-CF-PHs and HepG2 cells aggregated at 1 day and formed spheroids with diameter of approximately 100  $\mu$ m at 7 days (Fig. 4C and D). CF-PHs of other donors such as 16F2 were also able to form spheroids.

12M-CF-PHs in spheroids were subjected to double immuno-staining for ALB and CK7 (or CK19). They were both ALB<sup>+</sup> and CK7<sup>+</sup> (also CK19<sup>+</sup>) (data not shown). ALB secretion was quantified during culture (Fig. 5). The secretion level from 4 to 7 days increased 2.6-fold in monolayers as compared to that from 1 to 4 days, whereas the

level increased 6.6-fold in spheroids.

The above results suggested that spheroid-culture induces CF-PHs to express differentiated phenotypes. To further demonstrate such effects of spheroid-culture, the expression level of mRNAs of hepatocyte-differentiation marker proteins were compared between monolayer- and spheroid-cultures. This comparative study was performed using C-T PHs. Thus, before such experiments, gene expression profiles of C-T PHs was investigated in relation to those of fresh PHs. Total RNAs were extracted from C-T and fresh PHs of 3 donors, and used for quantifying mRNA of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ALB, HNF4, and cytochrome P450 isozymes (CYPs) (Fig. 6A). mRNAs of GAPDH, CYP2C9, and CYP2C19 in C-T PHs were expressed at a similar level as in fresh PHs. The expression of CYP2D6 and CYP3A4 in C-T PHs was higher than in fresh ones, whereas that of ALB, HNF4, CYP1A1, and CYP1A2 was lower in C-T PHs.

C-T PHs were prepared from 12M. Aliquots of them were used for total RNA extraction and the remaining was subcultured to 3 passages. CF-PHs at 3 passages and HepG2 cells as a reference cell were cultured in monolayers and spheroids for 7 days, and used for total RNA extraction. These RNAs were used to measure mRNAs of GAPDH, ALB, HNF4, AFP, and CYPs by real-time RT-PCR. The expression level of GAPDH mRNA was similar among monolayer- and spheroid-cultures of both CF-PHs and HepG2 cells, which was higher than that of the original (C-T PHs) level (Fig.6B). CF-PHs in monolayer expressed mRNAs of ALB (Fig. 6C) and HNF4 (Fig. 6D) at low levels as compared to the original level. In contrast, their expressions were significantly elevated to levels comparable to the original C-T PHs' level when cultured in spheroids. CF-PHs in both of monolayer-and spheroid-culture did not express AFP mRNA as in the case of C-T PHs (Fig. 6E). HepG2 expressed AFP mRNA at a high level. The original C-T PHs variably expressed mRNAs of CYPs (1A1, 1A2, 2C9, 2C19, 2D6, and 3A4) as shown in Fig. 6F. CF-PHs expressed them at very low levels in monolayer-culture. It was noteworthy that CF-PHs gained a mRNA expression profile of CYPs similar to that of the original C-T PHs in spheroid-culture. Such effects of spheroid-culture were not observed on HepG2 cells. They expressed mRNAs of ALB (Fig. 6C), HNF4 (Fig. 6D) and AFP (Fig. 6E) at a high level and expressed mRNAs of CYPs at an extremely low level (Fig. 6F) in both types of culture.

#### 4. Discussion

Previously, we cultured a human nonparenchymal cell (NPC) fraction in medium containing 3T3-CM, and showed that CF-PHs present in the NPC fraction can grow well [4]. In the present study we made primary cultures of human PHs in the presence of Swiss 3T3 cells and demonstrated the presence of CF-PHs, their occupancy rate being quite low. We were able to propagate them by serial subculturing.

In primary cultures of human PHs, we observed a small number of single cells started to divide at 5 days, grew slowly but steadily, and formed colonies. Such CF-PHs were characterized by phenotyping at earlier time points of culture. Most of the hepatocytes were immunocytochemically ALB<sup>+</sup> and CK19<sup>-</sup>. The primary hepatocyte population contained ALB<sup>+</sup> and CK19<sup>+</sup> cells at an occupancy rate of ~0.02%. The ratio of the number of colonies with ALB<sup>+</sup> and CK19<sup>+</sup> cells, and ALB<sup>-</sup> and CK19<sup>+</sup> to that of total colonies at 4 days of primary culture was 80 and 20%, respectively. The cells in all the colonies observed so far at 7 and 21 days in primary culture were ALB<sup>-</sup> and CK19<sup>+</sup>, strongly suggesting that originally ALB<sup>+</sup> and CK19<sup>-</sup> cells became ALB<sup>-</sup> and CK19<sup>+</sup> in the currently adopted culture conditions. Therefore, it is most likely that the biliary/progenitor cell features of CF-PHs are acquired during the periods of subculture. However, the possibility that ALB<sup>+</sup> and CK19<sup>+</sup> cells present at an occupancy rate of ~0.02% at the start expanded during culture can not be completely excluded at present.

Human CF-PHs were able to be subcultured within limited passages. CF-PHs from young donors could be passaged 7 times, PDT at passage 4 being 170-220 h, and, then the cells became senescent. CF-PHs from older donors were passaged only 5 times at most. They grew more slowly, their PDT at passage 4 being 650-1200 h. These results suggested that replication potential of human CF-PHs is correlated with the age of donors. It should be emphasized that the cumulative population doubling level (CPDL) of CF-PHs from younger donors, 3M and 12M, were as high as 19.3 and 24.7 during 300 days, respectively. Gibson-D'Ambrosio et al. serially passaged human PHs 12-15 times [9], using as medium supplemented with insulin, transferrin, hydrocortisone, EGF, and FBS. FBS was prescreened for their subculture, which was said to be important to obtain an optimal growth of the cells. Only 2 of the tested 7 FBS lots supported the growth of normal human PHs. PDT of PHs during subcultures was calculated as ~64 h. These PHs expressed ALB and CK18, but not AFP. At present, it is unclear whether

these proliferative hepatocytes are identical to the CF-PHs characterized in the present study. CF-PHs showed a liver epithelial cell-like morphology when they were in the growth phase and in an early phase of confluence, whereas they showed a typical hepatocyte-like morphology in condensed regions at confluence. CF-PHs expressed ALB, and CK7, 19, 8, and 18, but not  $\alpha_1$ -AT in the former conditions, indicating that these cells are able to express traits of both hepatocytes and biliary cells. In accordance with the morphological characteristics, CF-PHs in the condensed region expressed hepatocytic traits such as ALB, CK8 and 18, and  $\alpha_1$ -AT, but not biliary traits such as CK7 and 19. Thus, the CF-PHs seem to be hepatic progenitor-like cells existing even in the adult liver.

Previously, we devised HCGM as a culture medium that supports the growth of rat PHs [1]. Rat PHs grew in HCGM when co-cultured with NPCs [2] or Swiss 3T3 cells [6]. The growth promoting activity of 3T3 cells was partly replaceable with 3T3-CM. The active principles in 3T3-CM were HGF and pleiotrophin [6, 8]. In the present study we showed that 3T3-CM also supported the growth of human PHs, although its activity was less than that of 3T3 cells (data not shown).

A putative adult rat hepatocyte progenitor cells, Lig-8, elevated the expression of proteins of C/EBP $\alpha$ , ALB, and CYPs when cultured in spheroids [10]. We examined whether human CF-PHs in spheroid culture elevate differentiation-related genes. The mRNA expression level of ALB, HNF4, and CYPs of CF-PHs were low in monolayer-culture. However, when these CF-PHs were cultured in spheroid, they regained the expression of mRNAs of ALB and HNF4 to a level comparable to that of the original PHs, i.e., the cells before cultivation, and also regained a mRNA expression profile of CYPs of 2C9, 2C19, and 3A4 similar to that of original PHs. On the other hand, HepG2 cells expressed mRNAs of ALB and HNF4 even in monolayers to levels higher than those of PHs or cultured CF-PHs. In spite of such a high expression of HNF4 mRNA, HepG2 cells did not express CYP mRNAs in both monolayer- and spheroid-culture. Using an adenovirus-mediated antisense targeting method, a research group showed that the expression of CYPs in human PHs is regulated by HNF4 [11]. Currently, we suppose that human CF-PHs cultured in spheroids reexpress hepatocyte specific differentiation markers through the up-regulation of HNF4 expression.

In summary, the present study demonstrated the presence of CF-PHs, a highly replicative compartment of human PHs, even in the adult human liver. These CF-PHs

can be serially subcultured, retaining their normal hepatocytic characteristics. These cultures should be useful to study the cellular and molecular mechanisms of growth, differentiation, toxicity, metabolism, and carcinogenesis of human hepatocytes.

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### Figure legends

**Fig. 1.** Phase contrast microphotographs of PHs in culture. Thawed 9MM PHs were seeded at  $8 \times 10^3$  cells/cm<sup>2</sup> in 35-mm dishes (growth area, 9.6 cm<sup>2</sup>) and co-cultured up to 20 days with  $4 \times 10^3$  Swiss 3T3 cells/cm<sup>2</sup> in h-HCGM (A-H). Freshly isolated 16F2-PHs were seeded at  $8 \times 10^3$  cells/cm<sup>2</sup> in 35-mm dishes, and cultured up to 33 days as in 16F1-PHs (I and J). The mark (\*) was made on the back surface of the dishes on day 1 of culture to monitor the growth of seeded cells. A cell enclosed by dotted line in A was selected and monitored for the growth through 20 days by periodically taking phase contrast microphotographs. **A.** Day 4. **B.** Day 5. **C.** Day 6. **D.** Day 7. **E.** Day 10. **F.** Day 13. **G.** Day 17. **H.** Day 20. Arrows in A to H point to some of Swiss 3T3 cells. The size of colony increased gradually during culture. The area of colony was 0.0037, 0.0262, and 0.0685 cm<sup>2</sup> at 13, 17 and 20 days, respectively. Magnification of A to G is the same as in H. Bar in H, 100  $\mu$ m. **I.** PHs at 20 days. **J.** PHs at 33 days. Magnification of I is the same as in J. Bar in J, 100  $\mu$ m.

**Fig. 2.** Growth ability of CF-PHs. **A.** Colony-forming efficiency of PHs in relation to the age of donors. PHs were prepared from 3M, 16F1, 16F2, 45F, 53M, 58F, 61F, 63F, 63M, 68F, and 72M2, and were seeded at the density of  $8 \times 10^3$  cells/cm<sup>2</sup>, except PHs from 16F1 which were seeded at  $4 \times 10^3$  cells/cm<sup>2</sup>, and cultured for 20 days as in Fig. 1. The colony-forming efficiency was determined as the ratio (%) of number of colonies formed at 20 days of culture to that of the seeded PHs and plotted against the donor's age. The solid line represents the statistically significant regression curve ( $y = -0.001x + 0.076$ ,  $r^2 = 0.714$ ). **B.** Serial subcultivation of CF-PHs. PHs from 5 donors (3M, 12M, 16F2, 63M, and 72M1) were seeded at the density of  $8 \times 10^3$  cells/cm<sup>2</sup>, and cultured as in Fig. 1. They were detached as CF-PHs from the dishes at appropriate time points when they became confluent and subjected to subcultures, the seeding density being at  $4 \times 10^3$  cells/cm<sup>2</sup> at each passage. Open rhombuses, 3M-CF-PHs; closed squares, 12M-CF-PHs; open circles, 16F2-CF-PHs; open triangles, 63M-CF-PHs; open squares, 72M1-CF-PHs. PDT at passage 4 of CF-PHs from 3M, 12M, 16F2, 63M, and 72M1 was 220.3, 170.7, 223.6, 649.4, and 1,162.0 h, respectively. Cultures of 3M-, 12M-, and 16F2-CF-PHs were terminated at passages marked by \*, because they failed to grow thereafter. Cultures of 63M- and 72M1-CF-PHs were terminated at passages marked by

\*\* , because PDT became large thereafter.

**Fig. 3.** Phenotypes of CF-PHs. 3M- and 12M-PHs were serially subcultured. 3M-CF-PHs at passage 2 were cultured up to 13 days when the cells were still in pre-confluence and subjected to immunocytochemical staining (A and B). 12M-CF-PHs at passage 3 were cultured for 27 days when the cells were confluent and were subjected to immunocytochemistry (C - I), in which double immunocytochemistry for  $\alpha_1$ -AT and CK19 was done in G - I. **A.** CK8-staining. Most cells were CK8<sup>+</sup>. **B.** CK19-staining. All the cells were CK19<sup>+</sup>. **C.** ALB-staining. Most cells are ALB<sup>+</sup>. Especially hepatocytes in condensed regions indicated with \* are strongly positive. \* is similarly marked in D - I. **D.**  $\alpha_1$ -AT staining.  $\alpha_1$ -AT<sup>+</sup> cells are present in condensed regions. **E.** AFP-staining. All the cells were AFP<sup>-</sup>. **F.** Negative staining for C, D, and E. Magnification of A to E is the same as in F. Bar in F, 100  $\mu$ m. **G.** Red color shows anti-human  $\alpha_1$ -AT rabbit antibodies visualized by goat Texas red-anti-rabbit IgG. **H.** Green color shows anti-human CK19 mouse antibodies visualized by goat FITC-conjugated anti-mouse IgG. **I.** Photographs of G and H are merged.  $\alpha_1$ -AT<sup>+</sup> cells are negative for CK19. Magnification of G and H is the same as in I. Bar in I, 100  $\mu$ m.

**Fig. 4.** Phase contrast microphotographs of CF-PHs and HepG2 cultured in monolayers and spheroids. **A.** 12M-CF-PHs at 3 passages were detached from dishes and inoculated at  $10^5$  cells/cm<sup>2</sup> in 35-mm wells and cultured for 7 days. They showed a typical hepatocyte-like morphology in condensed regions (arrows). **B.** HepG2 cells were similarly cultured in wells as in A. **C.** 12M-CF-PHs at 3 passages similarly cultured as in A, but on Matrigel. **D.** HepG2 cells were similarly cultured as in A, but on Matrigel. Magnification of A through C is the same as in D. Bar in D, 100  $\mu$ m.

**Fig. 5.** ALB secretion by CF-PHs in monolayer- and spheroid-culture. 12M-CF-PHs at 3 passages were harvested from dishes at 22-58 days in culture and were cultured in monolayers (ML, open bars) and spheroids (SP, closed bars) as in Fig. 4 up to 7 days. The cells were refilled with fresh media at 1 day and cultured for additional 3 days (Day 1-4). Then, the cells were refilled with fresh media and cultured further for 3 days (Day 4-7). Media were collected at 4 and 7 days, and were used to quantify ALB of Day 1-4 and Day 4-7, respectively. The level of ALB secretion was determined as ALB secreted

during 3 days in culture per 1  $\mu\text{g}$  protein of CF-PHs in culture. CF-PHs in monolayer and in spheroid secreted ALB at  $5.0 \pm 2.2$  and  $5.0 \pm 3.9$  ng/3 days/1  $\mu\text{g}$  cell protein through 1 to 4 days, and  $13.1 \pm 0.6$  and  $33.2 \pm 2.7$  ng/3 days/1  $\mu\text{g}$  cell protein through 4 to 7 days, respectively. Each value represents the mean  $\pm$  S.D. (n=3). \* represents the statistical difference at  $P < 0.05$ .

**Fig. 6.** Expression of mRNAs of ALB, HNF4, AFP, and CYPs in monolayer- and spheroid-culture of CF-PHs. **A.** Comparison of gene expression between fresh and C-T PHs. PHs were prepared from 3 different donors (51M, 53M2, and 68F), and their aliquots were subjected to C-T. Fresh and C-T cells were used to quantify mRNAs of GAPDH, ALB, HNF4, AFP, and CYPs by real-time RT-PCR. We calculated the mean ratio of copy number of these mRNAs of C-T PHs to that of fresh ones. Each value represents the mean  $\pm$  S.D. (n=3). **B** through **F.** mRNA expression in CF-PHs in monolayer- and spheroid-culture. Fresh 12M-PHs were subjected to C-T. Aliquots of C-T PHs were used for RNA extraction and the remaining was subcultured up to 3 passages. CF-PHs at 3 passages and HepG2 cells were cultured in monolayer (ML) and spheroid (SP) for 7 days and were determined for mRNA expression by RT-PCR. **B.** GAPDH mRNA. **C.** ALB mRNA. **D.** HNF4 mRNA. **E.** AFP mRNA. **F.** CYPs mRNA. Values represent the mean  $\pm$  S.D. (n=3). \* and \*\* indicate statistical significance of difference by Student's *t*-test at  $P < 0.05$  and  $P < 0.01$ , respectively.

**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

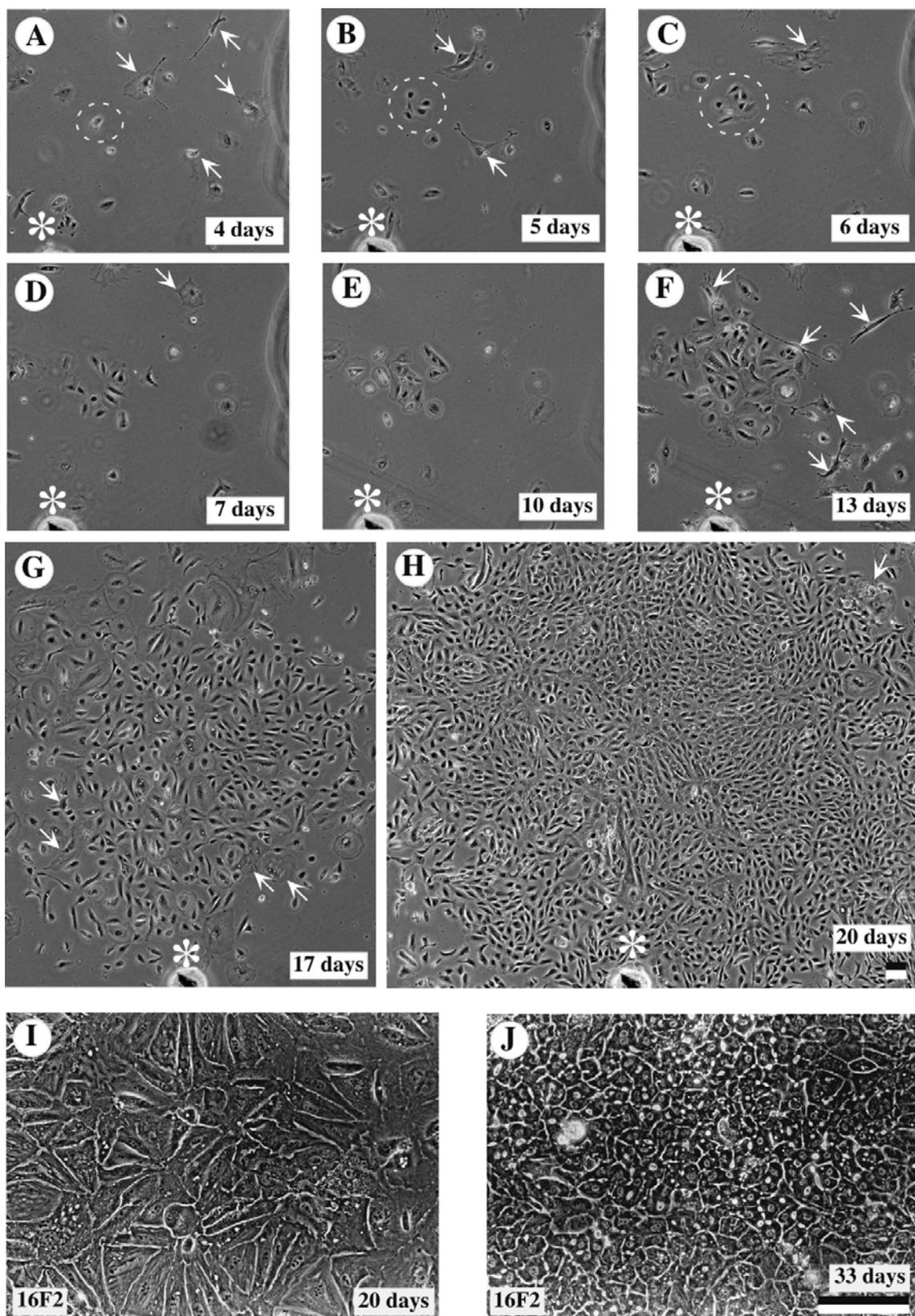


Fig. 1

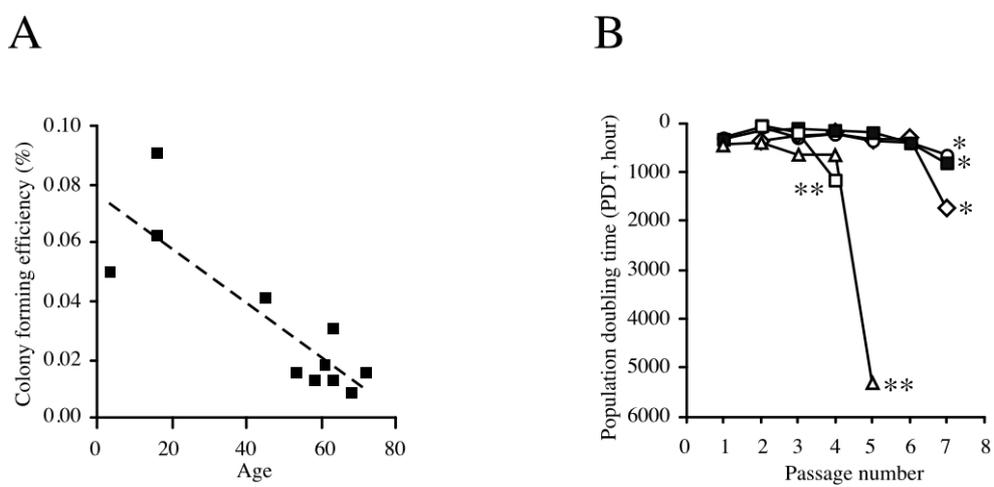


Fig. 2

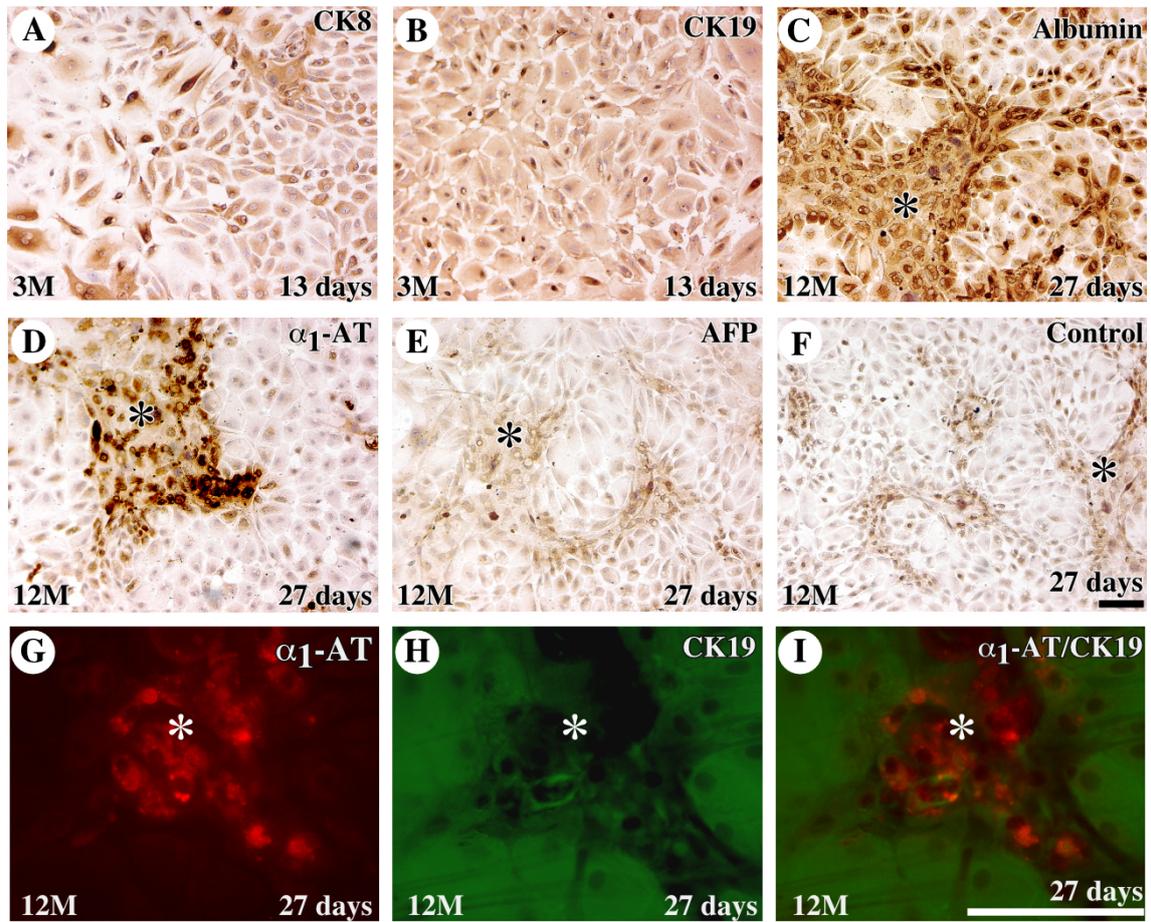
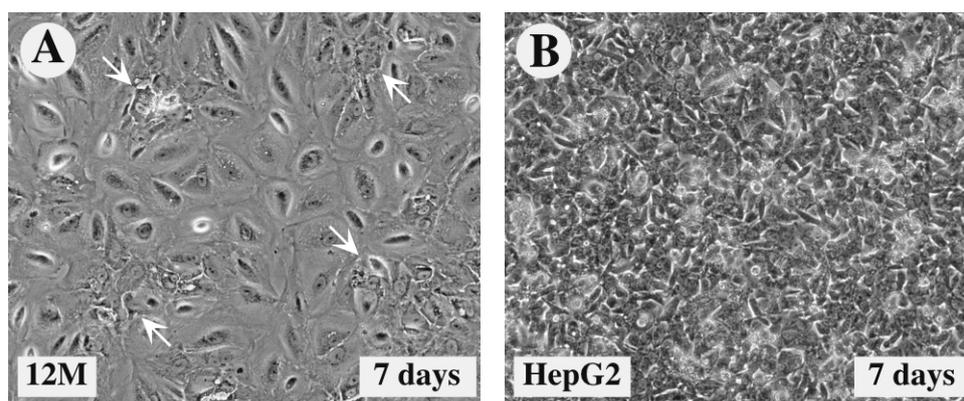
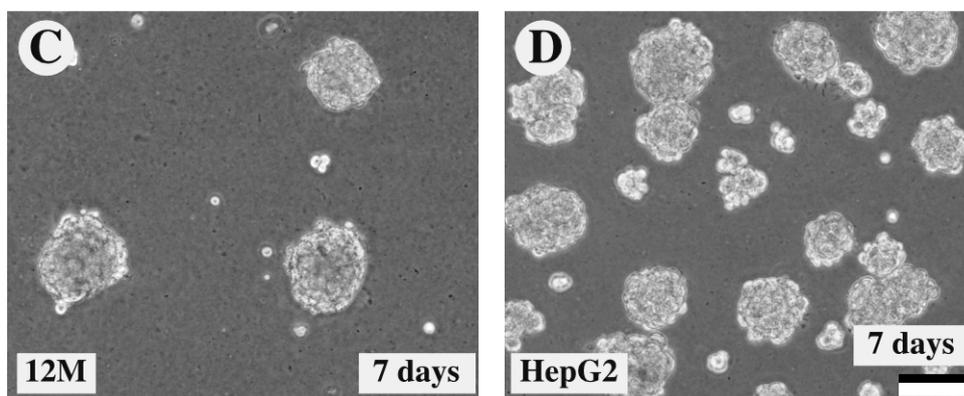


Fig. 3



**In dishes**



**On Matrigel**

Fig. 4

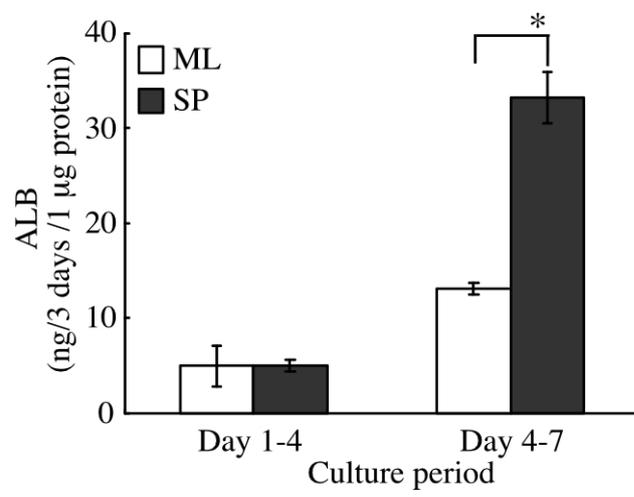


Fig. 5

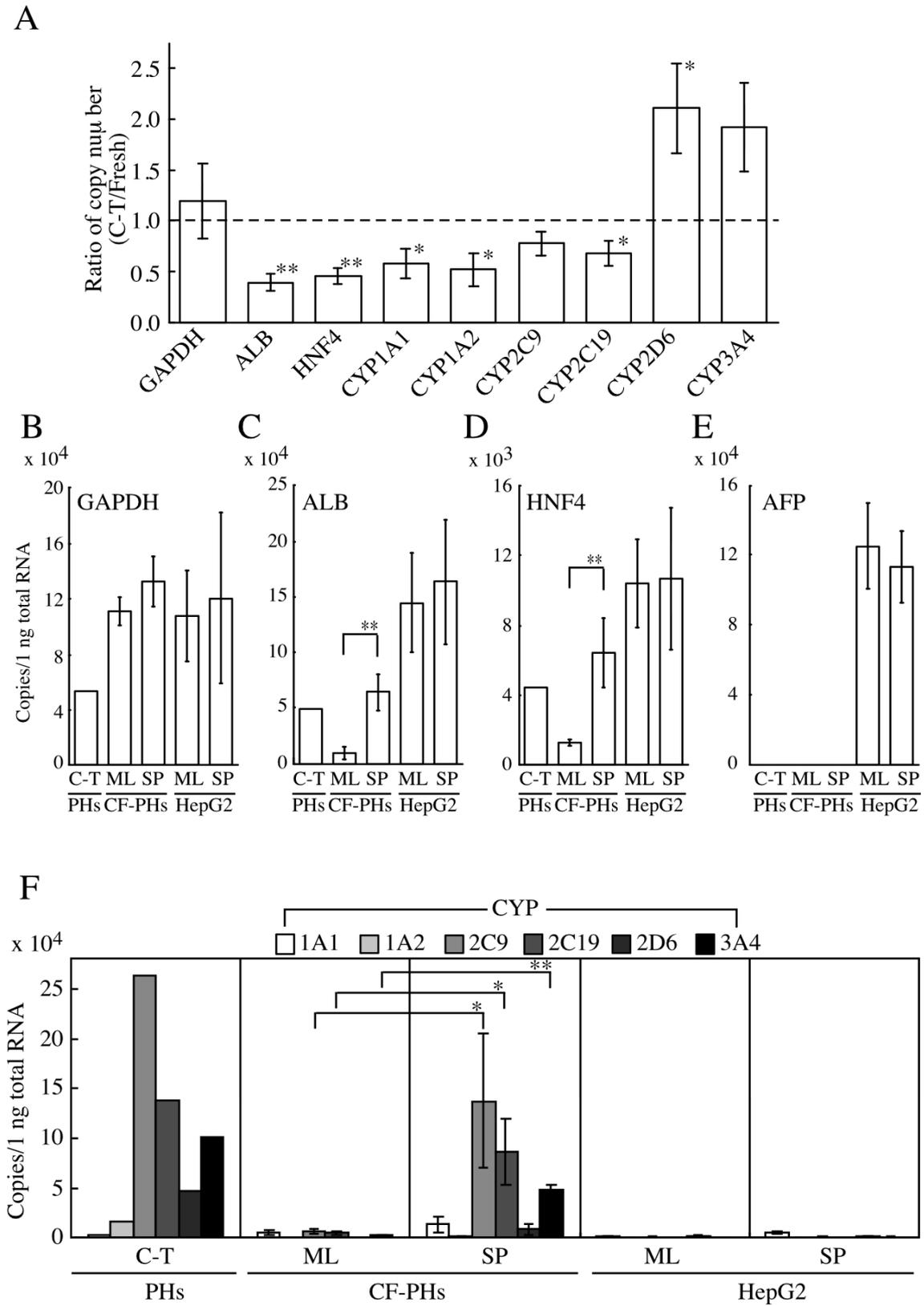


Fig. 6

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