Original Research

New and effective method to develop primary hepatocytes from liver cancer patients

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Impact Statement

Individualized tumor cell lines' *in vitro* applications in pharmacological and toxicological research are important. The primary human hepatocytes we established showed typical liver cell morphology and functions. More importantly, these cells can be established without genetic engineering or co-culturing with other cells. Some cells express NTCP, which means these cells might be susceptible to hepatitis B virus. The human hepatocytes developed can be used in cancer research, 3D culture, high-throughput screening, toxicity test, and individualized treatment.

Abstract

Liver cancer (LC) is one of the most common malignant tumors worldwide. Since the mechanism of LC pathogenesis and metastasis cannot be carried out directly on the human body, it is particularly important to establish human liver cancer cell lines for research *in vitro*. In this study, tissue block adherence method combined with cell clumps digestion method was used to establish primary human hepatocytes (PHHs) with a successful rate of 60% (45/75). Short tandem repeat (STR) analysis proved the cells were derived from its paired tissues. These cells from hepatocellular carcinoma (HCC) expressed NTCP and secreted ALB and AAT as detected by western blot, and expressed hepatocyte-specific membrane protein ASGR1 as detected by flow cytometry. Liver cancer biomarkers like CK7 in ICC (intrahepatic cholangiocarcinoma), AFP, and GPC3 in HCC expressed of different degree as detected by immunohistochemical analysis. These cells displayed typical liver cancer cell morphological characteristics and can passage stably. In conclusion, we developed an effective method to establish PHHs. Further studies are necessary to study if these cells maintaining other liver function and reproduce the physiology of the tumors and how these cells behavior in the drug development.

Keywords: Liver cell culture, primary human hepatocytes, 2D culture technologies

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Introduction

Primary liver cancer, ranked the sixth most common cancer and the fourth most common cause of cancer death worldwide in 2018,[1] is characterized by its pathologically heterogeneous and high rate of recurrence.[2] Hepatocellular carcinoma (HCC), which accounts for ~90% of primary liver cancers, is the most common primary liver cancer, and is the second lethal malignancy worldwide.[3] Infection with hepatitis B virus (HBV), hepatitis C virus (HCV), consumption of alcoholic, nonalcoholic fatty liver disease, aflatoxin B1 (AFB1) contamination of food, and hemochromatosis constitute of major risk factors.[4] In other words, all causes of cirrhosis may cause hepatocellular carcinoma.[4,5] Intrahepatic cholangiocarcinoma (ICC) is the second most common primary liver cancer, mainly caused by primary sclerosing cholangitis, cysts of the biliary duct, and parasitic infection with liver flukes.[3]

In the past decade or so, advances in precision medicine have brought hope to cancer patients with personalized treatment. However, effectiveness of treatment and drug development for liver cancer is still unsatisfactory due to the availability of easy and useful model systems that reproduce the function and physiology of the tumors in patients.[6] Establishment of primary liver cancer cell is difficult due to the changes of environmental signals in culture.[6] Furthermore, the quality of tissue is of great importance. The cell viability is affected by many factors including donor liver fat content, degree of liver damage, the time of tissue separation, and the method of treatment, for example, portal vein embolization showed no harmful effects on cell viability, however some

Table 1. Clinicopathological characteristics of PHHs.

Features	Mean value
Age	54.78 ± 8.82
	Number (*n*)
Gender (*n*)	
Male	62
Female	13
Clinical stage (*n*)	
1–2	63
3–4	12
Edmondson stage (*n*)	
I/I–II	17
II	27
II–III	23
III/III–IV/IV	4
Untested[a]	4
Tumor type (*n*)	
HCC	57
ICC	12
HCC&ICC	2
Untested[a]	4

PHH: primary human hepatocytes; HCC: hepatocellular carcinoma; ICC: intrahepatic cholangiocarcinoma.
[a]Totally necrosis of the tissues leads to untestable Edmondson stage and tumor type.

surgical manipulation, e.g. clamping during surgery, can lead to impaired cell viability.[7]

So far, many efforts have been made to develop *in vitro* liver cell culture models to address the need for drug development and individualized treatment. Great progress has been made during the last few years to better the cell culture models and improve stability and functionality of liver cells *in vitro*. These include genetic manipulation,[8] sandwich culture method,[9,10] and co-culture with fibroblast cell lines or non-parenchymal cells,[11–13] but there are few methods that can efficiently establish individual hepatocellular carcinoma cells and stably passage them *in vitro*. The current established hepatocyte tumor cell lines include HepG2, Hep3b, Huh7, and so on. The HepG2 and Hep3B cell lines were initially derived from tissue minces of hepatoma biopsies and cocultured with feeder layers of the irradiated mouse cell line STO.[14] The main limitation of these cell lines is the possibility of feeder cell contamination and relatively low expression of drug-metabolizing enzymes.[15]

Considering the advantages of individualized tumor cell lines' *in vitro* applications in pharmacological and toxicological research, establishment of *in vitro* liver cell model is getting more and more important. We now report the establishment of primary human hepatocytes (PHHs); these cells showed typical liver cell morphology and express typical hepatic functions. More importantly, these cells can be established without genetic engineering or co-culturing with other cells, for example, 3T3J2 and STO.[14]

Materials and methods

Patients and samples

The present study included 75 patients with primary liver cancer. The mean age of the patients was 54.78 years (range, 33–71 years), including 62 males and 13 females. Clinical stage, Edmondson stage, and tumor type were showed in Table 1. Samples contain 63 of clinical stage 1–2, 12 of clinical stage 3–4; 57 of HCC, 12 of ICC (intrahepatic cholangiocarcinoma), 2 of HCC&ICC, and 4 untested because of completely necrosis. Edmondson stage analysis showed samples are mainly moderately differentiated, rarely well or poor differentiated.

Isolation and establishment of PHHs

Tissues were obtained from patients undergoing primary liver cancer resection at the second hospital of Nanjing. Written informed consent was obtained from all patients. The study was approved by the ethics committee of the second hospital of Nanjing (2020-LS-ky016). The tissue (~0.05 cm^3) was maintained on ice and transferred to the laboratory as soon as possible (usually within 10 min). Tumor tissues were washed twice with DMEM (Gibco, Beijing, China) and minced with scissors into ~1 mm^3 pieces. The cells and small pieces of liver tissue were cultured in 25 cm^2 tissue culture flasks (Corning, Corning, NY, USA) with DMEM/F12 (Gibco, Grand Island, NY, USA) medium supplemented with 10% FBS (Gibco, Grand Island, NY, USA), 10 μmol/L γ-27632 (MCE, Billerica, MA, USA), 250 ng/mL Amphotericin B (Sigma, Darmstadt, Hessen, Germany), 100 ng/mL EGF (MCE, Billerica, MA, USA), 5 μg/mL insulin (Gibco, Grand Island, NY, USA). There would be several cell clone islands in 3–5 days. The cell clone islands were trypsinised and seeded in a six-well plate until cell confluency ~90%. Cells were passaged every 3 day (1/2 dilution) by trypsinization until cell confluency ~90%.

Western blot analysis

Western blot for liver-specific proteins was performed as described previously.[16] Tumor cells were harvested and lysed with RIPA buffer (KeyGEN BioTECH, Nanjing, Jiangsu, China), and protein concentration was quantified with a bicinchoninic acid protein assay kit (KeyGEN BioTECH, Nanjing, Jiangsu, China). The proteins were boiled with 5X loading buffer, and 40-μg aliquots of total protein were separated using 12.5% SDS-PAGE gels, prior to transfer onto PVDF membranes (EMD Millipore, Darmstadt, Hessen, Germany). The membranes were blocked at room temperature in 5% nonfat milk for 2 h and incubated with primary antibodies overnight at 4°C. Primary antibodies include anti-GAPDH (1:1000 dilution, cat.no. GB11002, Servicebio, Wuhan, Hubei, China); anti-Albumin (1:1000 dilution, cat.no. ab207327, Abcam, Cambridge, England, UK); anti-AAT (1:1000 dilution, cat.no. ab207303, Abcam, Cambridge, England, UK); anti-NTCP (1:2000 dilution, ab131084, Abcam, Cambridge, England, UK). The membranes were than washed and subsequently incubated with goat anti-rabbit IgG (H + L) secondary antibody (1:10,000 dilution, cat. no. SA00001-2, ProteinTech Group, Wuhan, Hubei, China) for 1 h at room temperature. Membranes were visualized using the FluorChem M System (ProteinSimple, San Francisco, CA, USA).

Population doubling time

About 3000 cells were plated into 96-well flatbottomed plates. CCK8 assay (MCE, Billerica, USA) was used to detect the

population doubling time and measured at 1 day intervals. WST8 activity was linearly related to the number of cells. The population doubling time was calculated according to the following formula:[17] doubling time (h) = h in culture * $\log_2 \div \log(A490^1/A490^2)$, in which $A490^1$ and $A490^2$ are the mean absorbance values of 3 wells from the CCK8 assay at the end and beginning of the time span measured, respectively.

Detection of HCC and ICC subsets by flow cytometry

Method to detect HCC and ICC was as our previous report.[18] Cells were labeled with FITC anti-human CD45 (ebioscienc, San Diego, CA, USA), PE-Cy7 labeled anti-EPCAM(CD326) (eBioscience, San Diego, CA, USA), and APC-labeled ASGR1 (mouse Monoclonal, MA1-40244, Invitrogen, Carlsbad, CA, USA), for 30 min at 4°C. Then, cells were washed and incubation with F(ab')2-goat anti-mouse IgG (H + L) secondary antibody, APC (17-4010-82, eBioscience, San Diego, CA, USA), for 30 min at 4°C. Then, cells were washed and detected in a FACS Canto II cytometer (BD, Franklin Lakes, NJ, USA). The data were analyzed by the FACSDiva (BD, NJ, USA) and FlowJo software (BD, NJ, USA).

Immunocytochemistry analysis

Cells and their corresponding tissues were analyzed with immunohistochemical method for liver tumor–specific biomarker. The expression of CK7, CK20 (Cytokeratin 7, Cytokeratin 20, both are cytoskeleton protein, used as biomarker of ICC), HEP1, GPC3, Arg1 (Hepatocyte paraffin 1, Glypican 3, Arginase-1, all are biomarker of HCC), and Ki67 (proliferation cell-associated nuclear antigen in tumor tissues) was assessed as reported before.[16] Cells were centrifuged at 1000 rpm for 5 min, and the pellet was embedded in paraffin for further immunohistochemical detection. The slices of tissue or cell were placed in an oven at 65°C for 1 h, then deparaffinized with xylene at room temperature for 3 times (10 min each) and then rehydrated with ethanol gradient treatment, including two rinses in absolute ethanol, a rinse in 90%, 80%, and 70% ethanol, respectively (7 min each), and finally three rinses in distilled water (3 min each). The slices were then immersed in boiling sodium citrate buffer for 5 min and naturally cooling down to room temperature. Following incubation in 10% BSA (Sangon, Shanghai, China) for 1 h at room temperature, the slices were incubated with primary antibodies at a dilution of 1:100 (anti-Ki67, Santa Cruz, CA, USA; anti-HEP1, biorbyt, Cambridge, UK; anti-GPC3, anti-CK7, CK20, anti-CD147, anti-ARG1, and anti-AFP are purchased from Abcam, Cambridge, England, UK) at 4°C overnight; this was followed by a further incubation with goat anti-mouse IgG (H + L) (ProteinTech Group, Wuhan, Hubei, China) or goat anti-rabbit IgG (H + L) (ProteinTech Group, Wuhan, Hubei, China) secondary antibodies at a dilution of 1:1000 at 37°C for 20 min. Following 5 min incubation with DAB and 2 min counterstain with hematoxylin and eosin (H&E), the slices were dehydrated and dried, and subsequently mounted with neutral gum. The slices were visualized using an Aperio ScanScope system (Leica Microsystems, Wetzlar, Hessen, Germany) and analyzed using Aperio Imagescope version 12.4.0.5043 (Aperio Technologies, Vista, CA, USA) for clinicopathological data of the tissues and cells.

Short tandem repeat analysis

Cells and its matched tissues were characterized using Short Tandem Repeat (STR) analysis by Genetic Testing Biotechnology, Suzhou, China. Nineteen short tandem repeat (STR) loci plus the gender determining locus, Amelogenin were amplified using the commercially available EX20 Kit from AGCU (Wuxi, Jiangsu, China). The samples were processed using the ABI Prism 3130 XL Genetic Analyzer. Data were analyzed using GeneMapper ID v3.2 software (Applied Biosystems, Foster City, CA, USA). Appropriate positive and negative controls were run and confirmed for each sample submitted.

Statistical analysis

Data analysis was performed using SPSS version 20 (SPSS Inc., Chicago, IL, USA). The χ^2 test was used to determine significant differences between the frequency of different categories. The independent sample *t*-test after Kolmogorov–Smirnov test was used to detect whether there is a difference between the mean value of age/rate of Ki67 expression of two groups. $P < 0.05$ was considered to indicate a statistically significantly difference.

Results

Successful establishment of PHHs

Seventy-five liver cancer tissues were harvested and cultured, 45 of which were successfully cultured, at a ratio of 60%. As shown in Table 2, among those successfully cultured, 91.1% (41/45) of specimens are from male patient, 73.3% (33/45) of specimens are of Edmondson stage II or more poorly differentiated, 80% (36/45) of specimens are HCC in tumor type.

Under the light microscope, cells displayed fusiform like or polygon morphology as shown in Figure 1(a). As is shown in H&E staining (Figure 2(b) and (c)), nuclei showed notable nuclear atypia, vary in size and shape, with round, quasi-circular and irregular shapes. The nuclear membrane is thickened, the nucleolus is obvious, the nucleolus is significantly eosinophilic, and mitotic figures are common.

PHH and its matched tissue were randomly chosen for STR analysis (Figure 1(c)). Nineteen STR loci and the Amelogenin locus were examined. Data showed the two submitted sample profiles are from human, but not a match for any profile in the DSMZ STR database. The DNA typing results support that the two submitted samples are from the same individual with a likelihood ratio of 3.3868×10^{21}. In other words, the tested cells are newly established. However, differences in the genotypes of three loci between the PHH and its matched tissue were observed. Interestingly, the genotypes of the three loci were D13S317(8, 13) in the cell, D13S317(8, -) in the tissue, D18S51 (12, 16) in the cell, D18S51 (12, -) in the tissue, FGA (21, 27) in the cell, and FGA (21, -) in the tissue. This is a good demonstration of the tumor heterogeneity: genotypes showed differences in some loci between the tissue used for STR detection and the tissue used to establish PHHs.

Table 2. Factors may affect the establishment success of the PHHs.

Features	Successful group (mean value/*n*)	Failure group (mean value/*n*)	*P* value
Age (mean value)	55.88 ± 7.95	53.13 ± 10.01	0.19
Degree of Necrosis (mean value)	0.1844	0.1733	0.854
Ki67 expression (mean value)	0.3748	0.4195	0.457
Gender (*n*)			
Male	41	21	0.0281
Female	4	9	
Total	45	30	
Clinical stage(*n*)			
1–2	36	27	0.3412
3–4	9	3	
Edmondson stage (*n*)			0.8248
I/I–II	9	8	
II	18	9	
II–III	13	10	
III/III–IV/IV	2	2	
Untested[a]	3	1	
Tumor type (*n*)			0.076
HCC	36	21	
ICC	4	8	
Other(untested[a]/HCC&ICC)	5	1	
Preoperative treatment (*n*)			> 0.99
Treated	19	13	
Untreated	26	17	

PHH: primary human hepatocytes; HCC: hepatocellular carcinoma; ICC: intrahepatic cholangiocarcinoma.
[a]Totally necrosis of the tissues leads to untestable Edmondson stage and tumor type.

PHHs express liver cancer–specific proteins

To identify whether the cultured cells express liver-specific proteins, western blot and immunohistochemistry were performed. Western blot showed that cultured cells express liver-specific proteins, such as human albumin (ALB), alpha 1 Antitrypsin (AAT), sodium taurocholate cotransporting polypeptide (NTCP, functional receptor for both HBV and HDV) (Figure 1(b)). According to immunohistochemistry assay, cultured cells showed obvious tumor cell characteristics, such as remarkable cytoplasmic and nuclear polymorphism, and pathological mitosis figures (Figure 2(b) and (c)). First, the tissues were assessed for tumor type. Then, the established matched cells were analyzed for liver cancer biomarkers. Ki67 expressed in deferent levels in PHHs and its original tissues (Figure 2(b) and (c)). CK7 expressed in the cytoplasm of PHH and its original tissue (ICC) (Figure 2(c)). GPC3, AFP are expressed of a different degree in the PHH and its original tissue (HCC) (Figure 2(b)). Differences were not observed between PHHs of different passages from histology analysis (Figure 2(b)). When compared with PHH from HCC and HepG2, we found that they share the same protein expression pattern (AFP+, GPC3+, Ki67+, HEP1-, Arg1-).

Cells express hepatocyte-specific biomarker: ASGR1

Tumor type of most tissues we used to establish PHHs was HCC according to pathological results. In the next, we detected the expression of epithelial cell adhesion molecule (EPCAM), which is a marker of bile duct cells, and asialoglycoprotein receptor 1 (ASGR1), which is a marker of hepatocyte in cultured cells. First, CD45+ (which is a marker of lymphocytes) cells were deducted (Figure 3(a)). The expression levels of EPCAM and ASGR1 were analyzed. Representative pictures showed that 81.7% PHH at P19 (EPCAM-ASGR1+) express hepatocyte-specific biomarker: ASGR1 (Figure 3(a)). In other words, the percentage of hepatocyte-origin cells in CD45 – subset is high. Results showed an average ASGR1 expression of 54.33% ± 17.75% (range = 23%–87%). As shown in Figure 3(a), the rate of ASGR1+ cells was 15.2% in passage 7, and 81.7% at passage 19, indicating that during the process of cell passage, the liver parenchymal cells gradually increase and become dominant population. With the subsequent passage of cells, the positive rate of ASGR1 of the cells is expected to be comparable with that of HepG2 (95.4%).

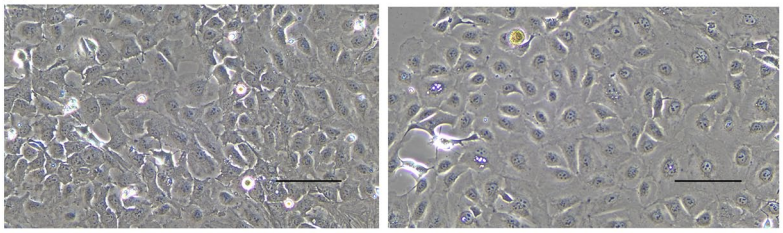
Cells can passage stably

Three PHHs were used for population doubling time analysis. All cells are continuous cell line and have been passaged *in vitro* for more than 50 generations. To avoid bias caused by long-term culture, cells passaged less than 35 times were used in the analysis. The population doubling time of PHH1 was 33 h, PHH2 was 53 h, and PHH3 was 43 h (Figure 3(b)).

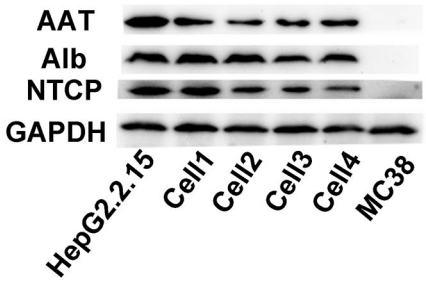
Factors affecting the establishment success of PHHs

Then, we analyzed the factors that may affect the establishment success of the PHHs including patients' age, gender, clinical staging, Edmondson differentiation grade, tumor type, preoperative treatment, degree of tissue necrosis, and expression of Ki67. As we can see in Table 2, there is a

(a)



(b)



(c)

Loci	A (Cell)		B (Tissue)		Matching Probability
Amelogenin	X	Y	X	Y	/
D3S1358	15		15		0.1225
D13S317	8	13	8		-
D7S820	8	11	8	11	0.0975
D16S539	10	12	10	12	0.0428
Penta E	15	16	15	16	0.0109
TPOX	8		8		0.2862
TH01	7	8	7	8	0.0203
D2S1338	18	19	18	19	0.0512
CSF1PO	11	12	11	12	0.1518
Penta D	9		9		0.0908
D19S433	12	14	12	14	0.0400
vWA	16	17	16	17	0.0756
D21S11	28	30.3	28	30.3	0.0005
D18S51	12	16	12		-
D6S1043	17	18	17	18	0.0170
D8S1179	13	14	13	14	0.0988
D5S818	11	12	11	12	0.1518
D12S391	17	18	17	18	0.0336
FGA	21	27	21		-
LR					3.3868×10^{21}

Figure 1. (a) Cell (from HCC, passage 3) images under the light microscope. (b) Cultured cells (from HCC, passage 6, 8, 5, 11) express the liver-specific proteins AAT, ALB, and NTCP. Protein was isolated from PHHs, HepG2.2.15, and MC38. Protein from HepG2.2.15 used as positive control and protein from MC38 used as negative control. AAT, ALB, and NTCP were detected by western blot. (c) STR analysis of cultured cells (from HCC, passage 5) and its matched tissues. Nineteen STR loci and the Amelogenin locus were examined.
LR: likelihood ratio; PHH: primary human hepatocytes.
Scale bars are 100 μm.

significant difference in the success rate of PHH establishment between male and female patients. No differences were observed in the success rate of PHH establishment among different age, degree of necrosis, Ki67 expression, clinical stage, Edmondson stage, tumor type, and preoperative treatment groups.

Discussion

The average age of patients in this study is 54.78 ± 8.87 years, mainly ⩾ 50, accounting for 70.66% (53/75), which is consistent with previous literature reports.[19] There was no significant difference in the age between the successful cell establish group and the unsuccessful cell establish group. Our data showed a significant difference in the gender between the successful cell establish group and the unsuccessful cell establish group. However, it is not clear whether this difference is caused by the small number of female specimens. It is well known that the incidence of liver cancer was higher in men than that of women (2.38% and 0.85%, respectively).[20] The clinical stage of the successful cell establish group mainly distributed in clinical stage 1–2. The main reason is that the clinical stage of the patients undergoing surgery are mainly stage 1 or 2, then stage 3, and the least stage 4. It is well known that patients in stage 4 are not suitable for surgery because the cancer have already metastasized. As we can see from the Table 2, whether the cell culture is successful or not is not dependent on age, clinical stage, Edmondson differentiation grade, tumor type, preoperative treatment, the degree of tissue necrosis, the positive rate of ki67 in the tissue. But the number of specimens in different Edmondson differentiation grade and intratumor heterogeneity may affect the

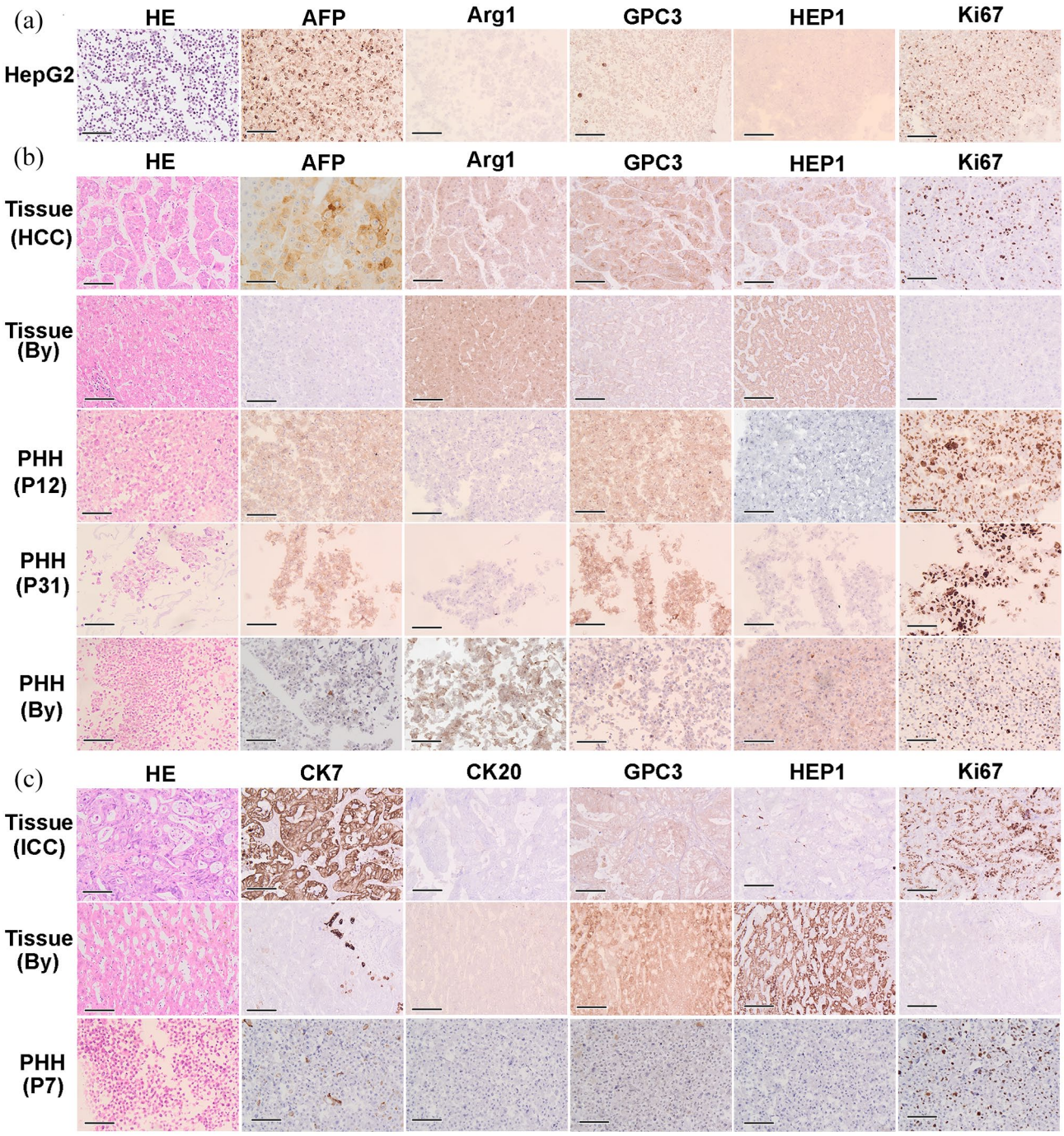


Figure 2. Histopathological characteristics of PHHs. (a) Specific proteins expression detected by immunohistochemistry on HepG2. (b) Specific proteins expression detected by immunohistochemistry on PHH original from HCC and paracancerous. (c) Specific proteins expression detected by immunohistochemistry on PHHs original from ICC. (A color version of this figure is available in the online journal.)
PHH: primary human hepatocytes; HCC: hepatocellular carcinoma; ICC: intrahepatic cholangiocarcinoma; By: paracancerous.
Scale bars are 100 μm.

results. As for preoperative treatment, generally interventional therapy or TACE (Transarterial Chemoembolization). Preoperative TACE, external radiation, and other preoperative treatments may promote tumor downgrading so that some patients get the opportunity of surgical resection. Some scholars believed that liver cancer patients who were resected after preoperative would have better long-term survival.[21] However, there were also report found preoperative TACE did not improve the patient's survival.[22] These reports give support to our results.

It is interesting that STR analysis showed differences in three loci. This is a good demonstration of the tumor heterogeneity: the tissue used for STR detection and the tissue used to establish PHHs displayed different genotype. Intratumor heterogeneity has been reported frequently, scientist observed intratumor heterogeneity ranging from 12.9% to 68.5% (at an

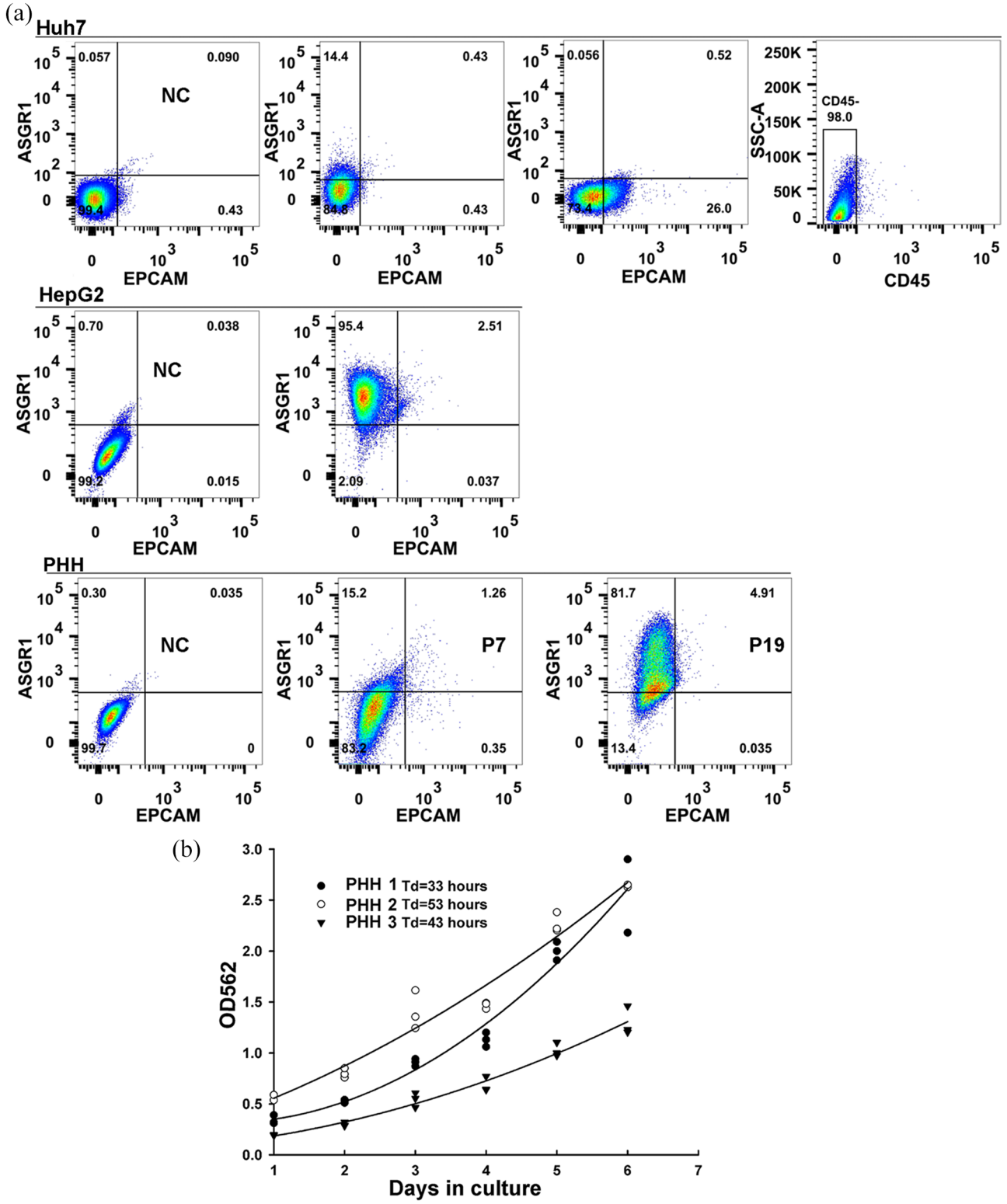


Figure 3. (a) Detecting hepatocyte-specific membrane protein ASGR1 expression by flow cytometry method. Huh7 and HepG2 cell lines were used as control. The PHHs original from HCC were identified as ASGR1 + EPCAM-CD45- (passage 7 and 19, respectively). (b) Growth curve of 3 PHHs (passage 25, 15 and 31, respectively) as determined by CCK8 assay. The cell population doubling time (Td) of PHH 1 was 33 h and that of PHH 2 and PHH 3 were 53 h and 43 h, respectively. (A color version of this figure is available in the online journal.)
NC: negative control; P: passage.

average of 39.7%) in all the 10 tumors detected.[23] Similarly, researchers reported that 26 of 30 tumor specimens from four tumors showed divergent allelic-imbalance profiles and two tumors in four showed ploidy heterogeneities.[24]

The liver is a complex organ originating from the endoderm and mesoderm. The liver is composed of two major type of cells including parenchymal cells and non-parenchymal cells. Parenchymal cells originate from the epithelium,

including hepatocytes and cholangiocytes, and constitute 70% and 3%–5% of total liver cells, respectively. Non-parenchymal constitute 25% of total liver cells, consisting mainly of Kupffer cells (KCs), hepatic stellate cells, and liver sinusoidal endothelial cells (LSECs),[6] play supportive roles to the liver parenchymal cells so as to maintaining liver function.[7] ASGR is a marker of hepatocyte and highly expresses in the liver parenchymal cells at their plasma membrane, responsible for the clearance of glycoproteins bearing N-linked glycans terminating with the structure Galβ1, 3GalNAc from the hepatocyte.[25] ASGR consists of two subunits ASGR1 and ASGR2. EPCAM is a marker of bile duct cells. As we can see in Figure 3, the cells showed 81.7% positive of ASGR1, which prove that these are liver-derived cells. What's more, these cells express liver-specific genes ALB, AAT and HBV/HDV receptor NTCP. The expression of the NTCP protein in our cells is comparable with that of HepG2.2.15, more efforts need to be made to address if these cells susceptible to HBV infection.

The conventional hepatocytes culture mainly refers to 2D culture. For over a century, 2D culture has been widely used as a tool to research tumor cell behavior and biology including cell proliferation, differentiate, migration, invasion, and so on.[26] Human hepatic cell lines are frequently used in culture models *in vitro* due to their advantages of good availability, high proliferation capacity, and the stable metabolism.[7] Although 2D culture of hepatocytes may lose part of the functions of hepatocytes, the good availability, high proliferation potential, and low cost make it a seed resource bank. Once the microenvironment rebuilds, the functions of hepatocytes can be quickly restored.[7] Although not been tested yet, our PHHs may lose part of the functions of hepatocytes. Many strategies could be used to improve the function performance of our PHHs, such as genetic modification or direct optimize the microenvironment favoring cell differentiation and liver-typical cell assembly *in vitro*.

The data from immunohistochemistry demonstrated that HCC-derived PHHs and HepG2 express similar proteins including AFP, GPC3, and Ki67. Similarly, the flow cytometry results showed that both HCC-derived PHHs and HepG2 displayed high expression of ASGR1 and far less expression of EPCAM. The positive rate of ASGR1 in passage 7 of PHH is 15.2% and rises to 81.7% in passage 19, indicating that with the passage of cells, liver parenchymal cells became the dominant population and gradually approached the ASGR1 expression level of HepG2. Western blot showed that PHHs (passage 6, 8, 5, 11) expresses ALB, AAT, NTCP, and previous study shows that HepG2 expresses ALB, AAT.[14] But the expression of NTCP in PHH drops rapidly according to reports.[27] The susceptibility of PHH to HBV is worthy of further research. When compared with cells that originate from paracancer tissue, cells that originate from HCC showed higher Ki67, AFP, and GPC3 expression. Correspondingly, cells originate from paracancer more likely reach replicative senescence[28] after limited cell divisions (usually within 10 passages).

In summary, we have developed a method to establish PHHs effectively without genetic manipulation or co-culturing with other cells. These cells express hepatocyte-specific membrane protein ASGR1 and can secrete ALB and AAT, can passage stably, and the population doubling time is comparable with ordinary cell lines. What's more, these cells express NTCP, which means these cells might be susceptible to HBV. However, further studies are still necessary to study if these cells maintaining other liver function, reproduce the physiology of the tumors, and how these cells behavior in the drug development. Taken together, using this method can build a seed resource bank for the research of liver function and step back and say, it helps building more satisfactory PHHs.

AUTHORS' CONTRIBUTIONS

All authors participated in the design and interpretation of the studies, the analysis of the data, and the review of the manuscript. YXY, LLW, and JYL were responsible for the design of the study. JYL, LLW, JF, and DXL conducted the experiments and collected the data. JYL, DDY, and YXY drafted the manuscript and LLW revised the manuscript. YFZ and JBH provided methodological and technical guidance. All authors read and approved the final manuscript.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

ETHICAL APPROVAL

The study was approved by the ethics committee of the second hospital of Nanjing (2020-LS-ky016).

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