

Human Liver Progenitor Cell Lines Are Readily Established From Non-Tumorous Tissue Adjacent to Hepatocellular Carcinoma

Aibin Zhang,¹⁻³ Roslyn London,² Frederik Michael Schulz,² Philippe Wilfred Roger Giguère-Simmonds,² Luc Delriviere,⁴ Harsha Chandraratana,⁴ Kathy Hardy,⁵ Shusen Zheng,¹ John K. Olynyk,^{3,6,7} and George Yeoh^{2,3}

Non-tumorous liver tissue removed during surgery to resect hepatocellular carcinoma (HCC) is potentially a useful source of material from which cells, particularly liver progenitor/stem cells (LPCs), can be isolated to establish cell lines. The purpose of this study was to evaluate the applicability of the “plate-and-wait” method to derive LPCs from resections to remove HCC. Three independent non-tumorous liver samples from HCC resection and 3 samples from liver donors were used for LPC isolation. Staining for LPC markers, OV6, CK19, and EpCAM, in the above liver samples demonstrated staining in only 2 of the non-tumorous samples. We isolated 2 human liver epithelial cell lines (HLECs) from these 2 samples. These HLECs were positive for general stem cell markers CD133, EpCAM, and Oct4. They expressed the liver progenitor cell markers OV6, CK14, and M2PK but not CK19. They also expressed the hepatocellular markers albumin, CK8, CK18, HNF4- α , and the drug-metabolizing gene *CYP3A4*. These cells accumulated glycogen, indocyanine green, and synthesized urea. They produced colonies in soft agar that showed anchorage-independent growth and their tumorigenic status was confirmed when they produced tumors following transfer to athymic nude mice. In contrast, the third non-tumorous tissue and 3 normal liver samples did not produce cell lines. This study establishes a correlation between the presence of LPCs in the source liver tissue and the ability to derive cell lines from these tissues. The phenotypic similarities between the LPCs and the HLECs suggest that a precursor-product relationship may exist between the 2 cell types.

Introduction

THE PRINCIPAL TREATMENT MODALITY of end-stage liver disease is orthotopic liver transplantation. The paucity of available organs is driving research to seek alternative therapies. One approach is cell therapy, and the use of hepatocytes has been evaluated with limited success, partly due to their fragility that makes them difficult to isolate and store. Liver progenitor/stem cells (LPCs) offer the advantage of being extremely robust, rapidly propagated and able to generate both hepatocytes and cholangiocytes. LPCs offer a major therapeutic opportunity in fields as diverse as gene therapy, initiation of liver repair, and biological liver support

systems. To realize this potential, it is necessary to identify suitable sources of LPCs, establish reproducible methods for their isolation, and define conditions for their expansion and subsequent culture, differentiation, and maturation into functional hepatocytes and cholangiocytes.

Several attempts have been made to isolate LPCs from diseased adult human liver [1–6]. To date, only one liver progenitor cell line has been established from a cirrhotic liver cancer patient following chronic HCV infection [4]. This cell line progressively exhibited polarized and functional hepatocytes and bile duct-like cell features under defined culture conditions. Other studies report the establishment

¹Department of Hepatobiliary Surgery, The First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, China.

²School of Biomedical, Biomolecular & Chemical Sciences and ⁷School of Medicine & Pharmacology, University of Western Australia, Crawley, Australia.

³The Centre for Medical Research, Western Australian Institute of Medical Research, Perth, Australia.

⁴Sir Charles Gairdner Hospital, Perth, Australia.

⁵Cytolabs Cytogenetics, Bentley, Australia.

⁶Department of Gastroenterology, Fremantle Hospital, Fremantle, Australia.

of cell cultures from human liver; however, it is not known whether cell lines were subsequently derived. Selden et al. [5] isolated epithelial cell colonies from explanted human liver from a patient with subacute liver failure. These cells exhibited hepatocyte, biliary epithelial, and stem cell markers and secreted albumin and α -1-antitrypsin. Crosby et al. [1] isolated human progenitor cells that differentiated only into cells with the phenotype of biliary epithelial or endothelial cells, as evidenced by expression of CK19 and CD31, but were negative for albumin. In contrast, Herrera et al. [3] isolated a liver stem cell population that expressed albumin and α -fetoprotein; however, these cells were negative for CK19. In vitro, these cells could be differentiated into mature hepatocytes. Recently, Duret et al. [2] isolated an epithelial cell population that did not express the classic set of stem cell/progenitor markers, but they could differentiate into hepatocyte-like cells. Weiss et al. [6] purified a cell population from adult liver with histological signs of regeneration as evidenced by Thy-1-positive cells. These were able to differentiate into hepatocytes in vitro and in vivo. In the current study, we evaluated the suitability of liver samples obtained from donor liver for orthotopic transplantation as well as from patients undergoing resection for hepatocellular carcinoma (HCC) as sources of LPCs. Using the "plate-and-wait" method described by Strick-Marchand et al. [7], which we have successfully applied to mouse liver [8–11], we established 2 cell lines from 3 non-tumorous liver tissue obtained from HCC patients. The cell lines expressed markers of stem cells as well as LPCs and some markers indicative of hepatocytes, but not cholangiocytes. However, these cell lines are tumorigenic; nevertheless, they are highly functional and may be useful for specific therapeutic applications.

Materials and Methods

Liver samples

The use of human liver samples and animal for scientific research has been approved by the Human and Animal Ethics Committees of The University of Western Australia, Sir Charles Gairdner Hospital and Fremantle Hospital, Western Australia. Care of animals was in accordance with institutional guidelines. Liver tissue samples were taken from 3 patients undergoing liver surgery for resection of HCC. The samples were (i) non-tumorous tissue approximately 4–5 cm from the HCC (NTT1, NTT2, and NTT3); (ii) tissue from an alternate lobe to that which contained the HCC (ALT2 and ALT3); and (iii) tumor tissue. Three liver tissue samples were obtained from donor livers prior to orthotopic transplantation and were termed normal tissue. Liver tissue was cut into blocks of ~ 0.5 cm³ and some used for cell isolation. The remainder was frozen for either RNA extraction or subsequent immunohistochemical staining.

Immunohistochemistry

Immunohistochemistry was performed on serial frozen sections (10 μ m) using monoclonal antibodies against cytokeratin (CK) 19 (Serotec, Raleigh, NC), OV6 (R&D, Minneapolis, MN), and epithelial cell adhesion molecule (EpCAM, Abcam, Cambridge, UK). These antibodies stain LPCs in human liver tissue [12,13]. The frozen sections were air-dried and fixed in methanol/acetone or paraformaldehyde. After blocking for

non-specific binding and intrinsic peroxidase activity, the sections were incubated with a primary monoclonal antibody overnight at 4°C. After washes, the sections were incubated with the secondary antibody (peroxidase-labeled; Bio-Rad, Hercules, CA) for 60 min. The staining reaction was developed with DAB substrate (DAKO, Glostrup, Denmark). Controls omitted either the first or the second antibody.

Cell isolation

The non-parenchymal cell population was isolated using the "plate-and-wait" method described by Strick-Marchand et al. [7]. In brief, 0.5-cm³ cubes of liver were chopped into fine pieces using a scalpel and treated with collagenase H (Invitrogen, Carlsbad, CA) at a concentration of 0.6 mg/mL for 20 min at 37°C. The collagenase H was discarded and the contents were dispersed in an Elvehjem Potter homogenizer in Williams' E medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS), 20 ng/mL epidermal growth factor, 30 ng/mL insulin-like growth factor II, 10 μ g/mL insulin, streptomycin, penicillin, and fungizone. This medium is referred to hereafter as growth medium. The cells were pelleted by centrifugation (1,000g for 5 min) and 5×10^6 cells were inoculated into 60-mm Petri dishes coated with collagen I and cultured in a humidified atmosphere with 5% CO₂ at 37°C. The growth medium was replaced on every second day. By repeated differential trypsinization and subculture, fibroblasts were gradually eliminated. Epithelial cell lines were established from dislodged colonies, and cultured in a fresh flask. Cells were dissociated with trypsin-EDTA and passaged every 3 days. Tissue samples from 2 HCC patients (NTT1 and NTT2) but not a third sample (NTT3) produced cell lines that were termed HLEC1 and HLEC2, respectively. They were maintained in medium supplemented with 5% FBS and unless stated otherwise cells between passages 8 and 16 were used. Their growth characteristics were determined using the Cellscreen System [14].

Karyotyping of the HLEC cultures

The cells were exposed to Colcemid solution (Invitrogen, Carlsbad, CA) for 12 h, lysed in 75 mM potassium chloride for 15 min, followed by fixation in methanol and glacial acetic acid (3:1 v/v) for 20 min at 4°C. Metaphases were prepared on glass slides and stained with Giemsa according to standard procedures. Karyotyping the HLEC cultures was achieved by the application of first principles of cytogenetic identification due to the massive alterations observed in these cell lines. Referral to the International System for Human Cytogenetic Nomenclature (ISCN) 2005 [15] could not be utilized as virtually no chromosomes were clearly identified as normal human. Chromosomes were arranged according to like structure and size, then assembled from largest to smallest, with the sex chromosomes placed at the bottom right of the karyotype.

Immunofluorescence analysis

Cultured cells were fixed with methanol/acetone (1:1 v/v) or 2% paraformaldehyde, and blocked with 5% goat serum in phosphate-buffered saline (PBS) for 30 min. Samples were incubated with primary antibodies overnight at 4°C

in a humidified chamber. After washes with PBS, samples were incubated with the secondary antibodies goat Alexa Fluor® 594 or goat Alexa Fluor® 488 (Invitrogen, Carlsbad, CA) in darkness for 1 h at room temperature. After washes, cells were mounted with ProLong® Gold antifade reagent with DAPI (Invitrogen). Huh7 cells and a fibroblast cell line were used as positive and negative controls, respectively. Negative controls that omitted the primary or secondary antibodies were also incorporated in all experiments.

RNA analysis

Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) and reverse transcription (RT) was performed with 2 µg of total RNA with random hexamers and ThermoScript RT-PCR Systems (Invitrogen) according to the manufacturer's protocols. PCR conditions were 95°C for 5 min; 95°C for 30 s, annealing temperature for 30 s, and 72°C for 30 s, for 30 to 35 cycles; 72°C for 10 min. DNA fragments were resolved on 2% agarose gels. Forward and reverse primers used for specific amplification of *CYP3A4* and *CK19* were: tgggatgttcattccaaa, tgtcctgttcttctgctgaa, and gccactactacgaccatcc, caaactgtgttcggaagtcat, respectively.

Periodic acid Schiff staining for glycogen

HLECs were fixed with 4% paraformaldehyde for 15 min at room temperature and stained according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO). HepG2 cells and a human fibroblast cell line served as positive and negative controls, respectively.

Cellular uptake and removal of indocyanine green

Indocyanine green (ICG) is an organic anion that is specifically taken up and eliminated by cultured hepatocytes [16]. To assess its uptake, 1 mg/mL of ICG (Sigma-Aldrich, St. Louis, MO) in growth medium was added to cell cultures. Following incubation at 37°C for 15 min, cells were rinsed 3 times with PBS and uptake of ICG was determined according to a previously described method [16]. To assess elimination, fresh medium was added and cultures re-examined 6 h later. HepG2 cells and a human fibroblast cell line served as positive and negative controls, respectively.

Urea production

Urea concentrations were determined by colorimetric assay (Roche Diagnostics, Indianapolis, IN). The quantity of urea produced was determined by harvesting medium supplied 24 h earlier to confluent cultures in 12-well plates. Cell numbers were determined by MTT assay. The hepatoma cell line HepG2 that has been reported to produce urea [17] was used as a positive control, and a human fibroblast cell line was used as a negative control. Fresh growth medium was used as a blank.

In vitro differentiation

In vitro differentiation was initiated by 2 methods. One is to culture in differentiation medium that induces LPC differentiation toward hepatocytes [8]. The second is to culture in Matrigel that promotes LPC differentiation toward cholangiocytes [7].

Growing epithelial cell colonies were allowed to reach confluence in growth medium and then exposed to differentiation medium. Medium was changed every 3 days. RNA was extracted 7 and 14 days after exposure to differentiation medium. Expression of *CPY3A4* of HLEC1 and HLEC2 was assessed by RT-PCR and compared with cells cultured in growth medium.

One milliliter of Matrigel (BD Biosciences, San Jose, CA) was suspended in 50 mL growth medium and frozen at -20°C overnight and then thawed at 4°C overnight to obtain a homogeneous 2% Matrigel medium. Cloned epithelial cells (5×10^5) were suspended in 3 mL of 2% Matrigel medium and plated onto 60-mm dishes. Expression of *CK19* in HLEC1 and HLEC2 was assessed in RNA extracted after 7 and 14 days culture and compared with cells before being cultured in Matrigel.

Soft agar and nude mouse tumorigenicity assays

Approximately 1×10^4 and 1×10^5 HLECs (p12-14) in 1.5 mL of 0.4% agar were plated over the solidified 0.8% agar layer. Cells were incubated in a humidified atmosphere with 5% CO₂ at 37°C and growth medium was added weekly to prevent drying. Anchorage-independent growth was assessed daily. For nude mouse assays, cells were grown to confluence in 35-mm diameter dishes (approximately 2×10^6 cells), washed with balanced salt solution, and harvested using a cell scraper. Cells were centrifuged (1,000g) and pellets were injected subcutaneously into BALB/c nude (nu/nu) mice ($n = 2$) that were 5 weeks old. Animals were examined daily for the formation of tumor until the experiment was terminated after 5 weeks. Tumors obtained were fixed in Carnoy's solution (60% ethanol, 30% chloroform, 10% acetic acid) for histology or snap-frozen for immunofluorescent staining as described above. Both assays were performed using tumorigenic PIL2 cells [10] as positive controls.

Results

Liver progenitor cells are present in human liver samples

Typical ductular reactions were observed in the specimens NTT1 and NTT2. All the neoductular structures in the portal triads of NTT1 and NTT2 contained cells that co-stained for CK19/OV6 (Fig. 1A and 1B). Many cells co-stained for CK19/EpCAM, or CK19/OV6 and therefore deemed to be LPCs. They were found scattered throughout the NTT1 and NTT2 samples (Fig. 1C-1F). In contrast, only a few cells positive for both CK19 and OV6 were observed in the portal tract regions of NTT3. There was no evidence of positive staining for both CK19 and OV6 in the surrounding parenchyma (Fig. 1G and 1H). A similar staining pattern was observed in ALT2 and ALT3 (Fig. 1I-1L). In normal liver derived from organ donors, the bile ducts contained cells that co-stained for CK19/OV6, and CK19/EpCAM. No double-stained cells for these LPC markers were present in the parenchyma (Fig. 1M and 1N).

Primary isolation and expansion of epithelial cells

Four different sources of liver tissue samples were used to isolate LPCs. From a total of 3 non-tumorous liver tissue

samples (NTT1–3) only 2 cell lines (HLEC1 from NTT1 and HLEC2 from NTT2) were isolated. Only one sample of liver tissue of suitable size was available from the alternative lobe (ALT3) for LPC isolation but failed to produce a cell line. Primary cultures derived from the 3 donor livers did not contain adherent epithelial colonies after 72–96 h. All cells in the primary culture from these 3 donor livers senesced after 3 months in culture. Similarly cultures established from the 3 samples of HCC tissue failed to generate epithelial cell lines.

In primary cultures derived from the liver samples NTT1 and NTT2, initial adherent clones appeared 72–96 h after

establishment. After 2 or 3 weeks, these colonies expanded and formed a confluent monolayer of cells. The morphology of the primary cultures was heterogeneous (Fig. 2A). By repeated subculture and selection for epithelial colonies, fibroblasts were eventually eliminated. HLECs appeared palmate at low density (Fig. 2B), and assumed a cobblestone arrangement of cells with a high nuclear-to-cytoplasm ratio at high density (Fig. 2C). HLECs exhibited an average doubling time of 25.6 h for HLEC1 and 26.4 h for HLEC2 (Fig. 2D).

HLECs were passaged >30 times over 6 months. Cytogenetic analyses showed that their karyotypes rapidly altered from metaphase to metaphase as they were passaged. The karyotype differences between early and late passage cultures suggest that the cells possessed chromosome instability (Fig. 3). A representative cell from each passage has been karyotyped to demonstrate evolution of the cell line through in vitro culture. HLEC1 and HLEC2 were similar to one another, while not being identical. The changes that occurred in both samples included additions and losses of whole chromosomes and derivative chromosomes. Some structural alterations were also identified (C3 in Fig. 3B; C5 in Fig. 3D). Table 1 summarizes the karyotypic changes observed in HLEC1 and HLEC2 when early and late passages are compared. The homogeneously staining region observed in the left-hand side chromosome labeled C5 in Figure 3A, 3B, and 3D is considered by cytogeneticists to represent tumorigenicity in oncology tissue culture and karyotyping.

Phenotype of HLECs

HLEC1 and HLEC2 displayed the same pattern and expression of markers tested and therefore, only the staining pattern of HLEC2 is shown (Fig. 4). HLECs expressed the general stem cell markers CD133 (Fig. 4A), EpCAM (Fig. 4B), and Oct4 (Fig. 4C). HLECs expressed highly the LPC markers OV6 (Fig. 4D), CK14 (Fig. 4E), and pyruvate kinase M2 (M2PK) (Fig. 4F). They did not stain positively for the LPC/cholangiocyte marker CK19 (data not shown). The HLECs also expressed hepatocyte markers CK8 (Fig. 4G), CK18

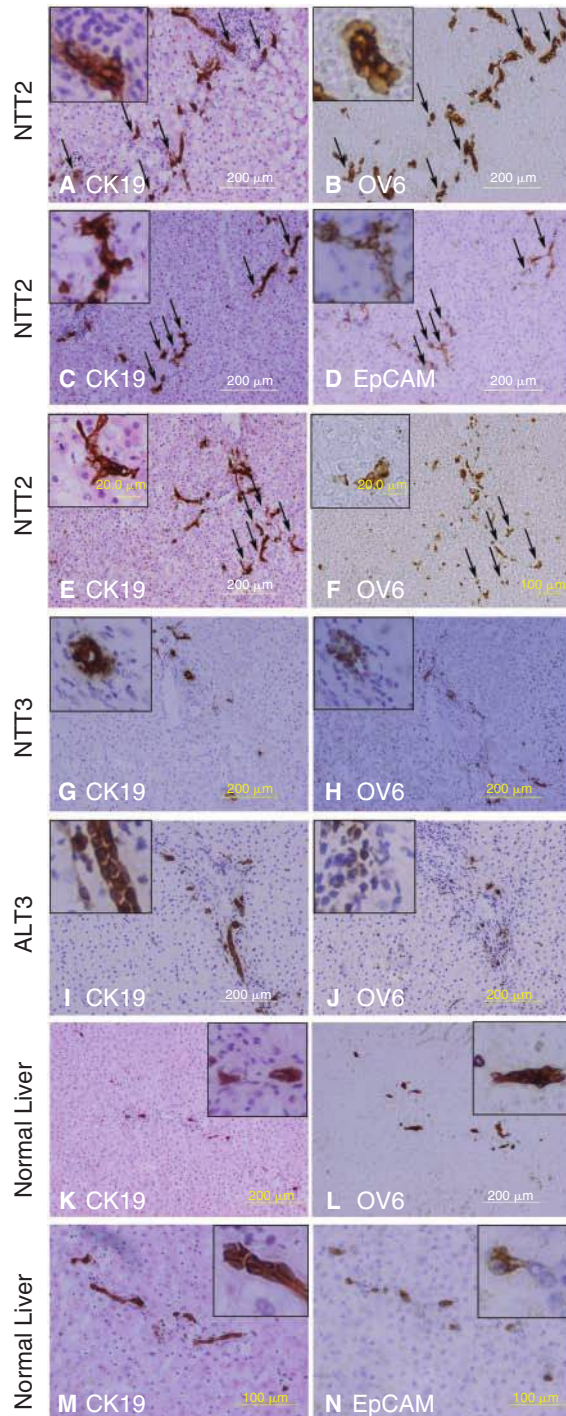


FIG. 1. Immunohistochemical staining of human liver sections. Typical ductular reactions were present in NTT2. The neoductular structures contained cells that co-stained for CK19/OV6 (arrows) (CK19 (A) and OV6 (B)). LPCs were revealed by double staining with either CK19/EpCAM or CK19/OV6 in serial sections. LPCs were not restricted to the portal tract, but were present in the surrounding parenchymal in NTT2 (arrows) (CK19 (C); EpCAM (D); CK19 (E); and OV6 (F)). In NTT3, very few cells in portal triad were double-stained by CK19 (G) and OV6 (H). No double-stained cells were observed in the surrounding parenchyma. A similar staining pattern was found in ALT3 (CK19 (I) and OV6 (J)). In normal liver, only bile ductular cells in the portal tracts were double-stained by CK19/OV6 and CK19/EpCAM. No double-stained cells were present in the parenchyma (CK19 (K); OV6 (L); CK19 (M); and EpCAM (N)). Scale bars are equal to 200 μm except in (F), (M), and (N), which is 100 μm . The inserted figures are 4 times the magnification of original image.

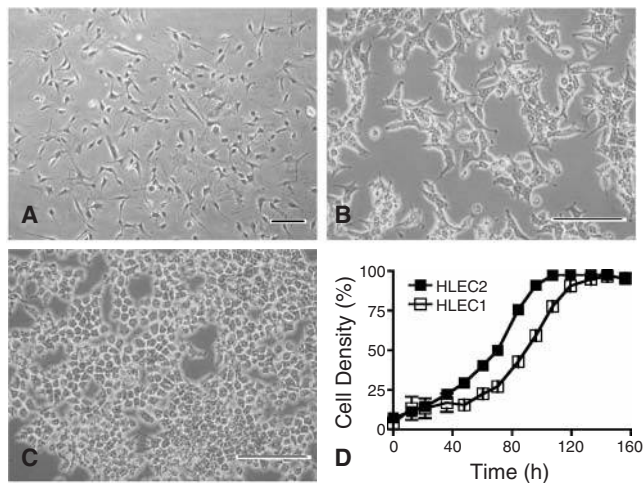


FIG. 2. Morphological characterization and growth properties of human liver epithelial cell lines (HLECs). Primary cultures from NTT1 and NTT2 tissue are heterogeneous (A). At low density, the HLECs appear palmate (B), while at high density, the cells assume cobblestone arrangement (C). HLEC1 and HLEC2 show similar growth characteristics (average doubling time of 25.6 h and 26.4 h, respectively) (D). Scale bar: 200 μ m.

(Fig. 4H), albumin (Fig. 4I), and hepatocyte nuclear factor 4- α (HNF4- α) (Fig. 4J). Human fibroblasts, used as a negative control, did not stain for any of these markers (data not shown). Huh7 cells were used as a positive control for OV6, CK19, CK18, Oct4, M2PK, and HNF4- α and were positive for all of these markers (data not shown).

HLECs possess hepatocyte functions

Both HLECs expressed *CYP3A4* (Fig. 5A). HLEC1 and HLEC2 secreted 207 fmol and 279 fmol of urea/cell/day, respectively, compared to 541 fmol of urea/cell/day by HepG2 cells (Fig. 5B). The quantity of urea in the medium harvested from the fibroblast cultures was below the threshold sensitivity of the assay.

HLEC2 stained positively with periodic acid Schiff (PAS) (Fig. 5C). HLEC2 demonstrated uptake of ICG (Fig. 5D) and eliminated the internalized ICG after 6 h (Fig. 5E). These properties were also displayed by HLEC1 (data not shown). HepG2 cells showed similar properties to HLEC1 and HLEC2, but not the human fibroblast cell line (data not shown).

In vitro differentiation conditions do not increase expression of CYP3A4 nor induce expression of CK19

Exposure to differentiation medium for 7 and 14 days did not increase expression of the hepatocytic marker *CPY3A4*. In Matrigel, HLEC1 and HLEC2 both formed a web-like structure throughout the dish (data not shown). Two to three days later, these became organized and developed into doughnut-like structures similar to bile duct units that have been described by Strick-Marchand et al. [7]. *CK19* was not detected in HLEC1 and HLEC2 by RT-PCR when these cell lines were maintained in growth medium. *CK19* expression remained negative after 7 and 14 days of establishment.

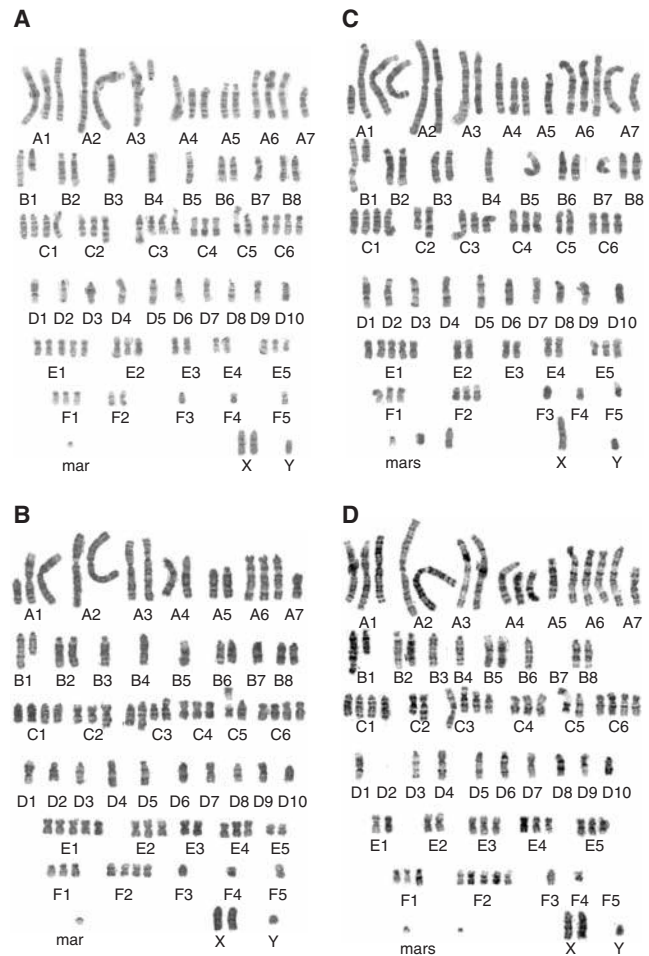


FIG. 3. Representative karyotypes of HLECs. HLEC1 p11 (A), HLEC1 p16 (B), HLEC2 p5 (C), and HLEC2 p9 (D) demonstrate both similarities and differences between passages and each other. Modal numbers are around 85 chromosomes, which differs from the normal diploid human with 46 chromosomes. The HLEC cells all demonstrate hypotetraploidy (<92 chromosomes), with many structural and numerical changes.

These findings were supported by immunofluorescence analysis (data not shown).

HLECs are tumorigenic

Both HLEC1 (data not shown) and HLEC2 (Fig. 6A) proliferated rapidly and formed large anchorage-independent colonies in soft agar after 2 weeks, similar to FRL-19 cells that served as positive controls. Subcutaneous injection of either HLEC1 or HLEC2 cells into athymic nude mice led to tumor formation within 3 weeks (Fig. 6B), similar to the tumorigenic PIL2 cell line. There was no apparent difference in the rate of growth of HLEC1 and HLEC2. The maximum tumor growth allowable by our animal ethics protocol of 1 cm in diameter was obtained on day 26 for mice injected with either HLEC1 or HLEC2. Tumors arising from HLECs were similar to the original cells injected into the mice with regard to morphology and phenotype, being small with scant cytoplasm (Fig. 6C) and exhibiting positive staining for CD133 (Fig. 6D) and EpCAM (Fig. 6E).

TABLE 1. SUMMARY OF THE KARYOTYPIC CHANGES OBSERVED IN HLEC1 AND HLEC2 WHEN AN EARLY PASSAGE OF EACH CELL LINE IS COMPARED WITH A LATER PASSAGE

HLEC1		HLEC2	
Chr #	Passage 11 vs. Passage 16	Chr #	Passage 5 vs. Passage 9
A1	Loss of short arm of chromosome on left	A1	Loss of derivative chromosome on left
A3	Additional copy of whole chromosome; loss of derivative chromosome on right	B5	Additional copy
A4	Duplication of chromosome on left; loss of two shorter chromosomes	B6	Loss of one copy
C3	Additional material translocated to the long arm of chromosome on left	B7	Loss of chromosome
E4	Additional copy	C3	Additional copy
E5	Loss of one copy	C5	Presence of homogeneously staining region in middle of chromosome
F2	Two additional copies	C6	Additional copy
		D2	Loss of chromosome
		E1	Loss of three copies
		E3	Gain of one copy
		E4	Gain of one copy
		F2	Gain of two copies
		mar	Loss of two markers and gain of one
		X	Gain of one copy

Abbreviation: Chr, chromosome.

Discussion

Liver tissue adjacent to HCC is a ready source of material from which liver cells can be isolated and maintained in culture. In the current study, we show that the “plate-and-wait” procedure described by Strick-Marchand et al. [7] which in our hands reproducibly generates LPC lines from mice [8–11], and readily produces epithelial cell lines from primary cultures established from non-tumorous human liver tissue that harbors LPCs. However, a cell line could not be established from a third non-tumorous liver tissue that did not show LPCs. One liver tissue sample taken from an alternate lobe to that which contained the HCC as well as 3 independent samples taken from donor livers prior to orthotopic liver transplantation, all of which did not display LPCs, also failed to generate epithelial cell lines. Thus, there is a correlation between the presence of LPCs and the ability to generate epithelial cell lines from HCC resection specimens.

Both HLEC lines that were established displayed some characteristics of stem cells as they expressed CD133, Oct4, and EpCAM and the LPC markers OV6, M2PK, and CK14. When maintained in growth medium, both cell lines express albumin, HNF4- α , and *CPY3A4*, and possess the ability to synthesize urea, store glycogen, and take up and release ICG. However, expression of *CK19* was not detected. Following culture under differentiation conditions, there is no increase in expression of hepatocytic marker *CPY3A4*, nor is there induced expression of *CK19*. Therefore, HLECs display a hepatocytic phenotype with functionality to match. We conclude that HLECs must have progressed some way along the hepatocyte lineage, and in this regard, they more closely resemble hepatoblasts with which they share the common feature of expressing EpCAM [18]. We are unable to say whether they attained this state in vivo, or in vitro.

This study shows that the “plate-and-wait” method readily produced HLEC lines from non-tumorous liver tissue that contained LPCs, as evidenced by immunohistochemical staining with a variety of established LPC markers. The HLECs were also positive for CD133 that numerous studies suggest is a marker of liver cancer stem cells [19–21]. In contrast, cell lines were not produced from the liver tissue adjacent to HCC in which immunohistochemical staining suggests there were no LPCs. Normal liver donor tissue prior to orthotopic liver transplantation were also not able to generate LPC cell lines and accordingly these did not harbor LPCs. Unexpectedly, we were unable to establish cell lines from the respective HCCs, whereas surrounding non-tumorous tissue produced HLEC1 and HLEC2. This suggests that LPCs capable of producing cell lines were not present in the HCC or they could not be selected for by the “plate-and-wait” method. Two tissue samples from an alternate lobe to that containing HCC did not exhibit LPCs in the parenchyma. Although one sample was too small to attempt establishment of a primary culture, the second was sufficiently large to do this, and failed to generate a cell line. We conclude that tissue adjacent to some HCCs harbors LPCs that are potentially tumorigenic, which can be used to generate HLECs using the “plate-and-wait” method. They may be cancer stem cells of the liver and may have migrated from the primary tumor, or they might have preceded the tumor. We are currently unable to distinguish between these possibilities. Such HLECs display extensive chromosomal alterations with increasing passage. While their tumorigenicity restricts their use in some therapeutic applications, their rapid proliferation and functional capacity in regard to drug metabolism and urea synthesis suggests that these robust and rapidly growing cells may nevertheless be useful in some therapeutic applications, such as bioreactors and other external liver assist devices for treating liver disease patients.

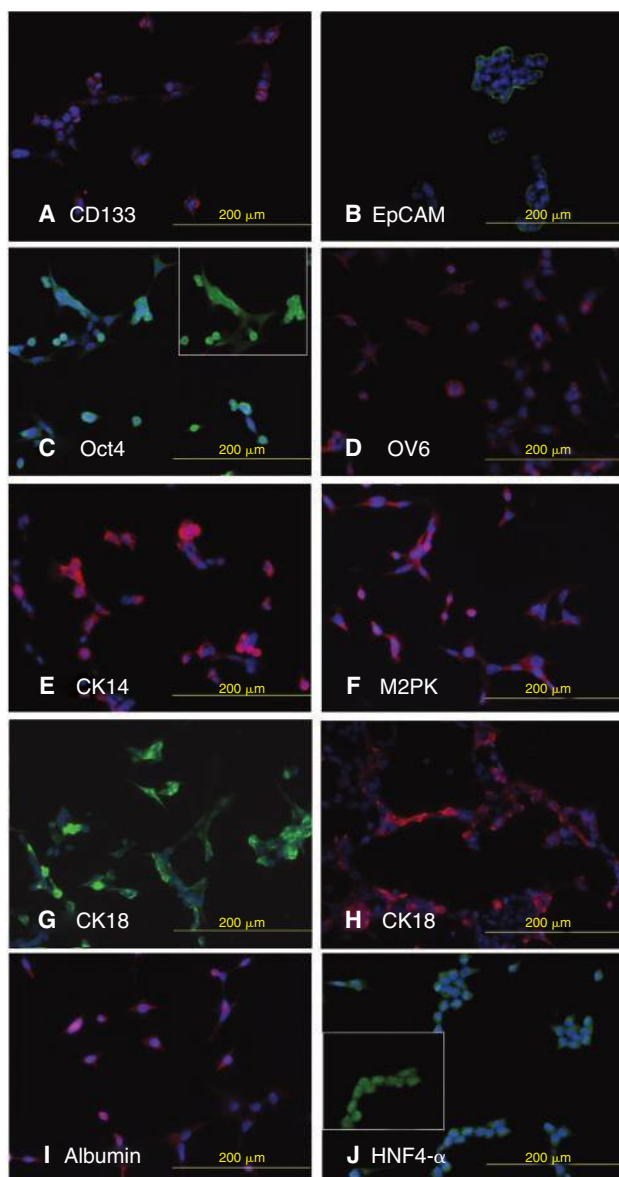


FIG. 4. Immunofluorescent labeling shows HLEC2 express the general stem cell markers CD133 (A), EpCAM (B), and Oct4 (C). HLEC2 highly express the LPC markers OV6 (D), CK14 (E), and M2PK (F). HLEC2 also express hepatocyte markers CK8 (G), CK18 (H), albumin (I), and HNF4- α (J). Nuclei are stained with DAPI (blue). CD133, OV6, CK14, M2PK, CK18, and albumin are stained with TRITC (A, D, E, F, H, and I). EpCAM, Oct4, CK8, and HNF4- α are stained with FITC (B, C, G, and J). The inserted figures in (C) and (J) show only FITC staining. Scale bar: 200 μ m.

Acknowledgments

This study was supported by National Health and Medical Research Council of Australia and The Cancer Council of Western Australia. John K. Olynyk is the recipient of a Practitioner Fellowship from the National Health and Medical Research Council of Australia. Aibin Zhang is a recipient of Training Fellowship from the National Health and Medical Research Council of Australia.

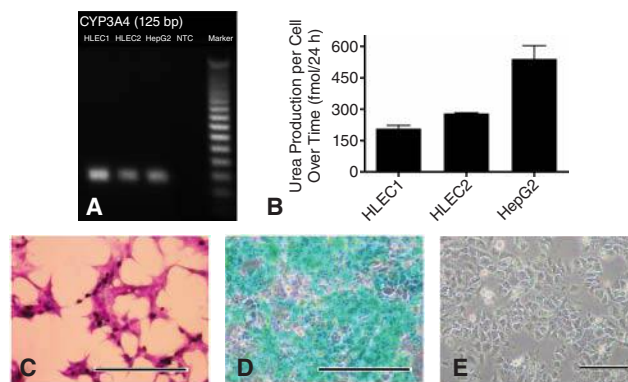


FIG. 5. HLECs exhibit features of hepatocytes. RT-PCR demonstrates *CYP3A4* gene expression in HLEC1 and HLEC2 (A). HepG2: positive controls; NTC, no template control. HLEC1 and HLEC2 synthesize urea (B). HepG2, positive control. Bars represent mean \pm SEM ($n = 3$). HLEC2 is PAS-positive indicating glycogen storage (C). HLEC2 takes up indocyanine green (D) and releases the dye after 6 h (E). Scale bar: 200 μ m.

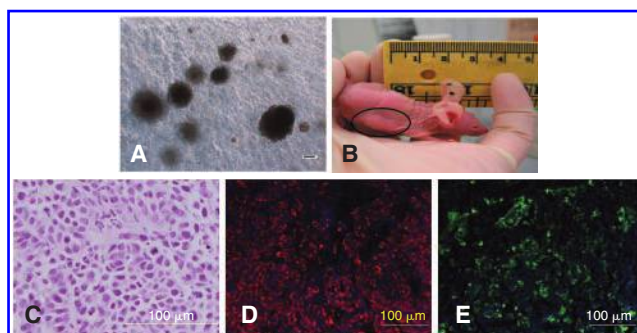


FIG. 6. HLECs are tumorigenic. HLECs form anchorage-independent colonies in soft agar (A). After injection of HLECs into a nude mouse, HLECs form tumors (B). H&E staining showed small cells with scant cytoplasm in tumor derived from HLEC2 (C). The tumor stained positively for the HLEC cell lines markers CD133 (D, TRITC staining) and EpCAM (E, FITC staining). Nuclei are stained with DAPI. Scale bar: 100 μ m.

Author Disclosure Statement

No competing financial interests exist.

References

- Crosby HA, DA Kelly and AJ Strain. (2001). Human hepatic stem-like cells isolated using c-kit or CD34 can differentiate into biliary epithelium. *Gastroenterology* 120:534–544.
- Duret C, S Gerbal-Chaloin, J Ramos, JM Fabre, E Jacquet, F Navarro, P Blanc, A Sa-Cunha, P Maurel and M Daujat-Chavanieu. (2007). Isolation, characterization, and differentiation to hepatocyte-like cells of nonparenchymal epithelial cells from adult human liver. *Stem Cells* 25:1779–1790.
- Herrera MB, S Bruno, S Buttiglieri, C Tetta, S Gatti, MC Deregibus, B Bussolati and G Camussi. (2006). Isolation and characterization of a stem cell population from adult human liver. *Stem Cells* 24:2840–2850.

4. Parent R, MJ Marion, L Furio, C Trepo and MA Petit. (2004). Origin and characterization of a human bipotent liver progenitor cell line. *Gastroenterology* 126:1147–1156.
5. Selden C, SA Chalmers, C Jones, R Standish, A Quaglia, N Rolando, AK Burroughs, K Rolles, A Dhillon and HJ Hodgson. (2003). Epithelial colonies cultured from human explanted liver in subacute hepatic failure exhibit hepatocyte, biliary epithelial, and stem cell phenotypic markers. *Stem Cells* 21:624–631.
6. Weiss TS, M Lichtenauer, S Kirchner, P Stock, H Aurich, B Christ, G Brockhoff, LA Kunz-Schughart, KW Jauch, HJ Schlitt and WE Thasler. (2008). Hepatic progenitor cells from adult human livers for cell transplantation. *Gut* 57:1129–1138.
7. Strick-Marchand H and MC Weiss. (2002). Inducible differentiation and morphogenesis of bipotential liver cell lines from wild-type mouse embryos. *Hepatology* 36:794–804.
8. Tirnitz-Parker JE, JN Tonkin, B Knight, JK Olynyk and GC Yeoh. (2007). Isolation, culture and immortalisation of hepatic oval cells from adult mice fed a choline-deficient, ethionine-supplemented diet. *Int J Biochem Cell Biol* 39:2226–2239.
9. Jellicoe MM, SJ Nichols, BA Callus, MV Baker, PJ Barnard, SJ Berners-Price, J Whelan, GC Yeoh and A Filipovska. (2008). Bioenergetic differences selectively sensitize tumorigenic liver progenitor cells to a new gold(I) compound. *Carcinogenesis* 29:1124–1133.
10. Dumble ML, EJ Croager, GC Yeoh and EA Quail. (2002). Generation and characterization of p53 null transformed hepatic progenitor cells: oval cells give rise to hepatocellular carcinoma. *Carcinogenesis* 23:435–445.
11. Lim R, B Knight, K Patel, JG McHutchison, GC Yeoh and JK Olynyk. (2006). Antiproliferative effects of interferon alpha on hepatic progenitor cells in vitro and in vivo. *Hepatology* 43:1074–1083.
12. Zhang L, N Theise, M Chua and LM Reid. (2008). The stem cell niche of human livers: symmetry between development and regeneration. *Hepatology* 48:1598–1607.
13. Lowes KN, BA Brennan, GC Yeoh and JK Olynyk. (1999). Oval cell numbers in human chronic liver diseases are directly related to disease severity. *Am J Pathol* 154:537–541.
14. Viebahn CS, JE Tirnitz-Parker, JK Olynyk and GC Yeoh. (2006). Evaluation of the “Cellscreen” system for proliferation studies on liver progenitor cells. *Eur J Cell Biol* 85:1265–1274.
15. Shaffer LG and N Tommerup. (2005). *ISCN2005. An International System for Human Cytogenetics Nomenclature*. Karger, Basel.
16. Yamada T, M Yoshikawa, S Kanda, Y Kato, Y Nakajima, S Ishizaka and Y Tsunoda. (2002). In vitro differentiation of embryonic stem cells into hepatocyte-like cells identified by cellular uptake of indocyanine green. *Stem Cells* 20:146–154.
17. Nyberg SL, RP Rimmel, HJ Mann, MV Peshwa, WS Hu and FB Cerra. (1994). Primary hepatocytes outperform Hep G2 cells as the source of biotransformation functions in a bioartificial liver. *Ann Surg* 220:59–67.
18. Itoh T, Y Kamiya, M Okabe, M Tanaka and A Miyajima. (2009). Inducible expression of *Wnt* genes during adult hepatic stem/progenitor cell response. *FEBS Lett* 583:777–781.
19. Rountree CB, S Senadheera, JM Mato, GM Crooks and SC Lu. (2008). Expansion of liver cancer stem cells during aging in methionine adenosyltransferase 1A-deficient mice. *Hepatology* 47:1288–1297.
20. Ma S, KW Chan, L Hu, TK Lee, JY Wo, IO Ng, BJ Zheng and XY Guan. (2007). Identification and characterization of tumorigenic liver cancer stem/progenitor cells. *Gastroenterology* 132:2542–2556.
21. Suetsugu A, M Nagaki, H Aoki, T Motohashi, T Kunisada and H Moriwaki. (2006). Characterization of CD133+ hepatocellular carcinoma cells as cancer stem/progenitor cells. *Biochem Biophys Res Commun* 351:820–824.

Address correspondence to:

Prof. George Yeoh
University of Western Australia
35 Stirling Highway
Crawley, WA 6009
Australia

E-mail: yeoh@cyllene.uwa.edu.au

Received for publication August 9, 2009

Accepted after revision October 29, 2009

Prepublished on Liebert Instant Online October 29, 2009

This article has been cited by:

1. Laia Tolosa, Ana Bonora-Centelles, M. Teresa Donato, Eugenia Pareja, Alejandro Negro, Silvia López, José V. Castell, M. José Gómez-Lechón. 2011. Steatotic liver: a suitable source for the isolation of hepatic progenitor cells. *Liver International* **31**:8, 1231-1238. [[CrossRef](#)]