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Original Articles

Establishment of a novel system for the culture and expansion of hepatic stem-like cancer cells

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ABSTRACT

Hepatocellular carcinoma (HCC) is a major primary liver malignancy in adults. Despite the progress made, the outcome of the treatment to this disease is less than satisfactory as the post therapy tumor recurrence is almost inevitable. Accumulating pieces of evidence have suggested that the recurrence is due to the existence of a subpopulation of the HCC cells that possess the properties of stem cells and are resistant to radiation and chemotherapy. It is therefore important to understand the characteristics of this subpopulation of HCC cells, and which requires the establishment of an *in vitro* system to study these stem-like cancer cells. However, despite extensive efforts, the progress in establishing such an *in vitro* system has been slow largely due to the lack of definitive biomarkers in the isolation and expansion of these cells. In order to successfully maintain and expand HCC CSCs, we first optimized the culture system. We establish a novel medium system that allows the culture and enrichment of these hepatic stem-like cancer cells from both hepatoma cells and human primary HCC cells. These cells exhibited typical stem cell properties, such as enhanced stem cell markers, gain of EMT properties and drug resistance, and more importantly, stronger tumor-initiating capabilities. The medium may help to establish an *in vitro* model for hepatic cancer stem cell (HCSC) studies, which may contribute to the development of novel cell therapies and new drugs for the treatment of HCC.

Introduction

Hepatocellular carcinoma (HCC) is the most common primary liver malignancy in adults. It is also the fifth most common solid cancer worldwide and the third leading cause of cancer related death [1,2]. Currently, surgical resection is the main option for the treatment of HCC, but unfortunately, due to resistance to both radiation and chemotherapy, postoperative tumor recurrence is almost inevitable. There are two theories that attempt to explain the formation of HCC: the classical clonal evolution model and the cancer stem cell (CSC) or tumor-initiating cell (TIC) model. The foundation of the TIC model of HCC formation is that HCC is a disease of adult stem cells. The model postulates the existence of a subpopulation of tumor cells exhibiting the stem cell properties of self-renewal and differentiation but also showing an innate resistance to radiation and chemotherapy. These cells are often referred as hepatic cancer stem cells (HCSCs) and they are defined by their ability to initiate tumor when transplanted into immune-compromised mice (transplantability) and by their capacity to recapitulate the heterogeneity of original cells in the primary lesions where they are derived from. The TIC model has recently received wide attention because it provides an explanation for resistance to therapeutic intervention due to the quiescent or slow cycling HCSCs that may survive the treatment such as radiation and/or chemotherapy and result in recurrence. This model is also supported by clinical observations and experimental evidence, as a subpopulation of HCC cells possesses two functional properties that are widely accepted to be associated with CSCs: resistance to radiation and chemotherapy and efficient transplant ability [3].

To better understand the etiology of HCC and develop effective anticancer approaches, there is a clear need of *in vitro* model systems for the study of the HCSCs. In the general CSC field, three types of *in vitro* models have been established [4]: (a) subpopulations selected from existing tumor lines; (b) cell lines created from tumor or normal cells by genetic manipulation; and (c) CSCs selected from tumors or sorted tumor cells using defined serum-free conditions.

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There have been ongoing efforts in identifying and characterizing these cells. In the last decade, several promising specific cell surface markers for putative HCSCs have been identified including CD133 [5], CD90 [6] and EpCAM [7]. These markers have been used to negatively or positively select for a subpopulation of HCC cells that are capable of initiating tumor in immune-comprised mice, but still possess the resistance to radiation and cytotoxic agents. However, these markers showed different expression patterns in different subsets of tumor-initiating HCC cells, suggesting the lack of definitive markers for HCSC selection and the heterogeneity of these HCSCs, which may be activated by different signaling pathways in normal stem/progenitor cells where these HCSCs may originate. Another method of HCSC isolation is based on the differential efflux of fluorescent dyes, such as rhodamine 123 or Hoechst 33342. However, as Hoechst 33342 is cytotoxic, the enrichment of tumor-initiation abilities of side population (SP) cells is most likely due to an artifact of Hoechst 33342 toxicity, because these SP cells are protected by their membrane transport properties; whereas non-SP cells are unprotected and unable to grow due to the toxicity. Thus, the enrichment of tumor-initiating SP cells is unlikely due to intrinsic stem-cell properties. Recently, the physical properties of the HCC cells were applied to select HCSCs, in which these cells were reported to be enriched by density gradient centrifugation [8]. However, the quantity of the cells obtained is limited and the cells cannot be further enriched with this method.

The selection of CSCs by using defined serum-free culture conditions is a powerful approach to create *in vitro* models for CSC expansion and differentiation [9–11]. The strength of this method is the ability to define the correct environment (niche) required to maintain the stemness of the CSCs. This method has been successfully used to select and expand cell populations with CSC characteristics from patients with colon and lung cancers [12] and gliomas [13]. In this study, we developed a defined serum-free medium that is able to enrich and expand HCSCs, maintain their stem cell traits but are still capable of tumor initiation. After extensive screening, we selected a formula that allows us to culture HCSCs from both hepatoma cells and primary human HCC cells. These cultured cells exhibited up-regulated HCSC biomarkers such as CD90, enhanced epithelial–mesenchymal transition (EMT) properties, invasive potentials and resistance to chemotherapy drugs. More importantly, the tumor initiation capability of these cells was significantly increased. Taken together, the medium developed in this study allows us to effectively enrich the population of HCSCs from both hepatoma and human primary HCC cells, which may facilitate the study on HCSCs.

Materials and methods

Primary tumor cell culture

The protocol was approved by the Institutional Review Board of the Eastern Hepatobiliary Hospital, Second Military Medical University, Shanghai, China. The HCC biopsies were provided by the Department of Liver Surgery, Eastern Hepatobiliary Surgical Hospital, Shanghai, China. Written consents were obtained from patients prior to sample acquisition. The tumor tissues were washed and minced into fragments of $1 \times 1 \times 1$ mm^3 before being digested in 0.5 mg/ml type-I collagenase at 37 °C for 20–30 min to make a single cell suspension. Cells were then washed with D. Hanks solution twice, and 1×10^5/ml cells were seeded in petri dishes with 2–3 ml DMEM supplemented with 10% fetal bovine serum (FBS) or defined serum-free media, and cultured in a 5% CO_2 incubator at 37 °C.

Cell line culture, sphere formation and passage

Human hepatoma cell lines Hep3B and Huh7 were obtained from the American Type Culture Collection (ATCC). MHCC97-L, MHCC97-H [14], and HCCLM3 [15] were generous gifts from Shanghai Zhongshan Hospital (Shanghai, China). All of the cells were maintained in DMEM with 10% FBS, 100 IU/ml penicillin G and 100 μg/ml streptomycin at 37 °C in a 5% CO_2 incubator. For suspension sphere culture, cells were first washed with PBS to remove serum, and then suspended in seven defined serum-free media with formulas summarized in Table S1. Tumor cells at different passages were plated at ultralow attachment 6-well plates (Corning Inc., Corning, NY, USA) at a density of 5000 cells/well. The formation of tumor-spheres was observed under an inverted light microscope at 100× and 200× magnifications. The spheres were collected by gentle centrifugation, then dissociated with trypsin–EDTA and mechanically disrupted with a pipette. The resulting single cell suspension was then centrifuged to remove the trypsin–EDTA and re-suspended in serum-free medium to allow the re-forming of spheres. The spheres were passaged every 5–8 days before they reached a diameter of ~100 μm.

Cell cycle assays

Cell cycle analysis was performed after fluorescence labeling of the cellular DNA with propidium iodide (PI) (Invitrogen, Carlsbad, CA, USA). After two rinses with cold PBS, HCC cells were harvested during the exponential growth phase, fixed with cold 70% ethanol, and then incubated at 4 °C for 4 hours. After centrifugation at 1000 rpm for 3 minutes and being rinsed with cold PBS, cells were resuspended in 1 ml PI stain (50 mM PI, 100 mg/ml RNase) and incubated at room temperature for 30 min. The cell cycle of the cells was then analyzed by FACS (BD, US).

Invasion assay

The invasive potentials of HCC and sphere-forming cells were compared by using BioCoat Matrigel invasion chambers (BD, Franklin Lakes, NJ) with 8 μm pore membrane filters in a 24-well culture plate by following the manufacturer's instruction. HCC cells were resuspended at a final concentration of 1×10^4 cells/ml in 0.1 ml DMEM containing 1% FBS in the upper chamber. The lower chambers were loaded with 0.6 ml DMEM containing 10% FBS. After 24 h or 48 h, cells that migrated through the Matrigel and attached to the bottom of the membrane were fixed and stained with crystal violet. Migrated cells in three different fields were counted. Three replicated experiments were performed and error bars represented the standard error of the mean.

In vivo *tumorigenesis assay*

The protocols for *in vivo* mouse xenograft model were approved by Medical Experimental Animal Care Unit of the Second Military Medical University. Six to eight week-old BALB/c nude mice were provided by Shanghai SLAC Laboratory Animal Co. Ltd of Chinese Academy of Sciences, and the mice were maintained in a pathogen-free condition. The defined number of parental and sphere-forming cancer cells mixed with 0.1 ml serum-free DMEM/Matrigel (1:1) was inoculated in the left or right flank of mice. Tumor growth was monitored every 3 days starting on the third week after the inoculation. The mice were sacrificed at day 90 or when the tumors grew to a maximum of 1000 mm^3. Tumor xenograft volume in mm^3 was calculated by the formula: Volume = $(\text{width})^2 \times \text{length}/2$.

Chemotherapy sensitivity assays

The sensitivities of the Hep3B and HuH7 parental and sphere-forming cells to chemotherapeutic drugs were measured by Cell Counting Kit-8 (CCK-8) assay using WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium. Briefly, 4000 cells/well were seeded in 96-well plates, and various concentrations of doxorubicin (Sigma-Aldrich) were added at the beginning. After 72 h incubation, viable cells were measured by CCK-8 assay by following the manufacturer's instruction.

Quantitative reverse transcription PCR (qRT-PCR)

RNA samples were extracted from both the parental and the sphere cells, cDNA were synthesized using the Superscript III first-strand cDNA synthesis kit (Invitrogen, Carlsbad, CA). qRT-PCR was carried out using SYBR Green PCR Master Mix. The PCR protocol was: 95 °C for 10 min, followed by 40 cycles of 95 °C 15 s, 60 °C 60 sec and 72°C 45 sec. The following primers were used: Wnt-1, 5′-TAAGCAGGTTCGTGGAGGAG-3′ (forward) and 5′-GGTTTCTGCTACGCTGCTG-3′ (reverse); CD90, 5′-TGAAGGT CCTCTACTTATCCGC-3′ (forward) and 5′-GCACTGTGACGTTCTGGGA-3′ (reverse). EpCAM, 5′-GCTGGGGGAGGGGAGCCTAC-3′ (forward) and 5′-ACTGCTCACCTCTGGCCGCT-3′ (reverse). NOTCH1, 5′-GTTGGGGTCCTGGCATC-3′ (forward) and 5′-GGTGAGA CCTGCCTGAATG-3′ (reverse). NOTCH2, 5′-CACAGGGTTCATAGCCATCTC-3′ (forward) and 5′-GGAGGCGACCGAGAAGAT-3′ (reverse). NOTCH3, 5′-CCTGAGTGACAGGGGTCCT-3′ (forward) and 5′-TGTGCAAATGGAGGTCGTT-3′ (reverse). Oct4, 5′-GAACCATACT CGAACCACATCC-3′ (forward), 5′-CGTTCTCTTTGGAAAGGTGTTC-3′ (reverse). Nanog, 5′-TTTGGAAGCTGCTGGGGAAG-3′ (forward), 5′-GATGGGAGGAGGGGAGAGGA-3′ (reverse). CD133, 5′-AGAGCTTGCACCAACAAAGTACAC-3′ (forward), 5′-AAGCA CAGAGGGTCATTGAGAGA-3′(reverse). CD44, 5′-TGCCGCTTTGCAGGTGTAT-3′ (forward), 5′-GGCCTCCGTCCGAGAGA-3′ (reverse). Beta-Actin, 5′-ATCTGGCACCACACCTTCTACAA-3′ (forward), 5′-GTACATGGCTGGGGTGTTGAAG-3′ (reverse). Relative gene-expression quantification method was used to calculate the fold change of mRNA expression according to the comparative C_t method using β-actin as an endogenous control. Final results were determined as: $2^{-(\Delta Ct\ \text{sample}-\Delta Ct\ \text{control})}$. Data were represented as ratio or fold changes to parental cells.

Immunofluorescence staining

HCC derived adherent cells were labeled with anti-human protein antibodies (see below) and fixed with 4% paraformaldehyde for 20 minutes. After blocking with 10% Bovine Serum Albumin (BSA) (Sigma Aldrich), cells were incubated overnight with primary antibodies: anti-human cytokertin 8/18 monoclonal antibody (Abcam), mouse antihuman E-Cad antibody (Abcam) and mouse antihuman vimentin antibody (R&D) and antihuman CD90 monoclonal antibody (Becton Dickinson, Franklin Lakes, NJ, USA) at the dilution of 1:100. Tyramide signal amplification kits (PerkinElmer Life and Analytical Sciences, Inc.) were used with Cy3 or Cy5 (red/green) fluorescence.

RNA sequencing and data mining

The RNA samples (~50 ng) were amplified with a MessageAmp II aRNA Amplification kit (Ambion) to generate multiple copies of amplified RNA (aRNA) by following the manufacturer's instruction. After amplification, aRNAs were purified and fragmented into small pieces. The cleaved RNA fragments were reverse transcribed into a single-strand cDNA, followed by second strand DNA synthesis with DNA polymerase I. The synthesized double-stranded cDNA fragments were subjected to purification, and then ligated to Illumina adapters using Quick ligation TM kit (NEB) and DNA ligase. The resultant cDNA adapter-modified cDNA libraries were amplified by 15 cycles of polymerase chain reaction. The cDNA libraries were finally subjected to cluster formation, primer hybridization and sequencing reactions using the Illumina Genome Analyzer II (Illumina, San Diego, CA, USA) by following the manufacturer's instruction. Raw short sequence fragments were accepted if they passed the quality filtering parameters used in the Illumina GA Pipeline GERALD stage.

High-quality reads were aligned to the human reference genome (NCBI Build 36.1) using NextGENe® software (Softgenetics, State College, PA, USA). The matched reads were aligned to Human Refseq mRNA (NCBI). Reads shorter than 20 bps and those with the quality score less than 14 were excluded. The sequences aligned with individual transcript were counted digitally. The expression levels for each gene were normalized to reads per kilobase of exon model per million mapped reads (RPKM) to facilitate the comparison of transcripts among samples. Large databases containing all gene transcripts identified by RNA-Seq for the samples of sphere-forming cells from Hep3B, Huh7 and MHCC97-H cell lines were assembled. A mean $\log_2$ fold change [RPKM of sphere-forming cancer cells/RPKM of parental cells] of each gene was calculated across all 3 cell lines. The false discovery rate (FDR, i.e. a probability of wrongly accepting a difference between parental and sphere-forming cancer cells) of each gene was determined according to the previously reported method [16]. The genes were regarded as differentially expressed when their FDRs were less than 0.05. Furthermore, genes were classified as up-regulated when their mean $\log_2$ fold change ratios were larger than 1 or down-regulated when their $\log_2$ fold change ratios were less than −1.

Statistical analysis

Data were collected using an MS-Excel spreadsheet. Group comparisons for continuous data were done with Student's t-test for independent means or two-way ANOVA. All experiments were performed in triplicate. Statistical significance was set at $P < 0.05$.

Results

Optimization of the culture medium and expansion of sphere-forming cells from various human liver cancer cells

In order to successfully maintain and expand HCC CSCs, we first optimized the culture system. After careful examination, seven efficient culture systems (Table S1) were selected to be further assessed. In these culture media, cells generally formed non-adherent, three-dimensional sphere clusters. We finally determined that C3 formula is the most suitable culture system, which contains DMEM/F12, Neurobasal-A medium, B27 without VA, EGF and the cytokines FGF-10 and IGF-1. By using the C3 medium, sphere-forming or stem cell-like cancer cells from Hep3B (named as Hep3B-C) (Fig. 1A) and Huh7-C (named as Huh7-C) (Fig. 1B) HCC cell lines were successfully cultured and expanded. Stem cells are characterized by their unique capability to keep self-renewal and differentiation. Herein, we determined the self-renewal capacity of sphere-forming cells by testing their capability for serial passage. The spheres formed from Hep3B/Hep3B-EGF cells after being cultured in C3 medium for 0, 10, 21, 23, 45, and 90 days were shown in Fig. S1, and the results showed that spheres were formed and the same status was maintained during the entire 3 month passage, indicating their self-renewal capability *in vitro*.

Next, we cultured an additional three HCC cell lines, HCCLM3, MHCC97-H and MHCC97-L, in the C3 medium. All of these cell lines were able to form spheres that were stable during a long-term culture. We named them as HCCLM3-C (Fig. 1C), MHCC97-H-C (Fig. 1D) and MHCC97-L-C (Fig. 1E). It is critical to culture and maintain stem cell-like cancer cells from primary tumor tissues for both diagnostic and therapeutic purposes. Herein, we also successfully established three cell lines with different characteristics from a primary HCC tumor tissue and they were named EHBH-HCSC-1 (Fig. 1F), EHBH-HCSC-2 (Fig. 1G) and EHBH-HCSC-3 (Fig. 1H). These cells grew well in the form of sphere for more than 3 months in the C3 medium. We also evaluated the cell origin of these CSC-like sphere-forming cancer cells by using glycogen staining (Fig. S2) and ICG uptake experiments (Fig. S3) [17], and the results showed that Hep3B-C and HuH7-C cells were originally from HCC cells. Taken together, these results demonstrated that the C3 culture system is suitable for general *in vitro* culture and maintenance of various cell lines and primary HCC cells that are of sphere-forming capability.

Differential expression of CSC biomarkers in sphere-forming cancer cells

Compared with parental cells, HCC stem cell-like cancer cells have unique characteristics as well as specific biomarkers. After the demonstration of tumor-derived cells to organize self-renewing spheroids, the expression of genes specific to tissue or embryonic stem cells was examined. To further characterize the CSC-like cells, we used qRT-PCR to detect the expression of CSC biomarkers such as CD90. The results showed that the mRNA expression level of CD90 (Fig. 2A), Oct4 (Fig. 2G), and Nanog (Fig. 2H) was ubiquitously up-regulated, while that of NOTCH1 (Fig. 2B), NOTCH2 (Fig. 2C), and NOTCH3 (Fig. 2D) were down-regulated in sphere-forming cells in comparison with that of parental cells. Generally, mRNA expression of Wnt-1 (Fig. 2E) and EpCAM (Fig. 2F) appeared to be down-regulated in most sphere-forming cells. We also analyzed the cell markers CD133 and CD44, the results are shown in the supplementary materials (Fig. S4). Flow cytometry analysis of $CD90^+$ and $EpCAM^+$ showed that the $CD90^+$ positive cells were significantly enriched by about 5-fold in Huh7-C, and 20-fold in Hep3B-C cells, while EpCam positive cells dramatically decreased in both cell lines (Table S2), which indicates that CSC-like cells were dramatically enriched in the cells cultured in the C3 medium.

Differential expression of epithelial–mesenchymal transition (EMT) markers in sphere-forming cancer cells

Recent studies indicate that the emergence of CSCs occurs in part as a result of the EMT, and the activation of the EMT process has been associated with the acquisition of stem cell traits by normal and neoplastic cells [18]. In this study, we compared these epithelial markers cytokeratins 8 and 18, as well E-cadherin and mesenchymal cell surface marker vimentin [19] in parental and sphere-forming cells by immunofluorescence. Results showed that the expression of cytokeratins 8/18 and E-cadherin were dramatically decreased, while vimentin was up-regulated in two sphere-forming cell lines, Huh7 (Fig. 3) and Hep3B (Fig. S5). Interestingly, while these sphere-forming cells were cultured in serum-supplemented medium, the expression of these proteins was restored to levels similar to that of parent cells, suggesting a differentiation process of these sphere-forming cells.

Sphere-forming cancer cells possess more invasive potential

Recent studies have identified the important role of CSCs in carcinogenesis and relapse. In several human cancers including HCC, studies showed that some subsets of CSCs were responsible for

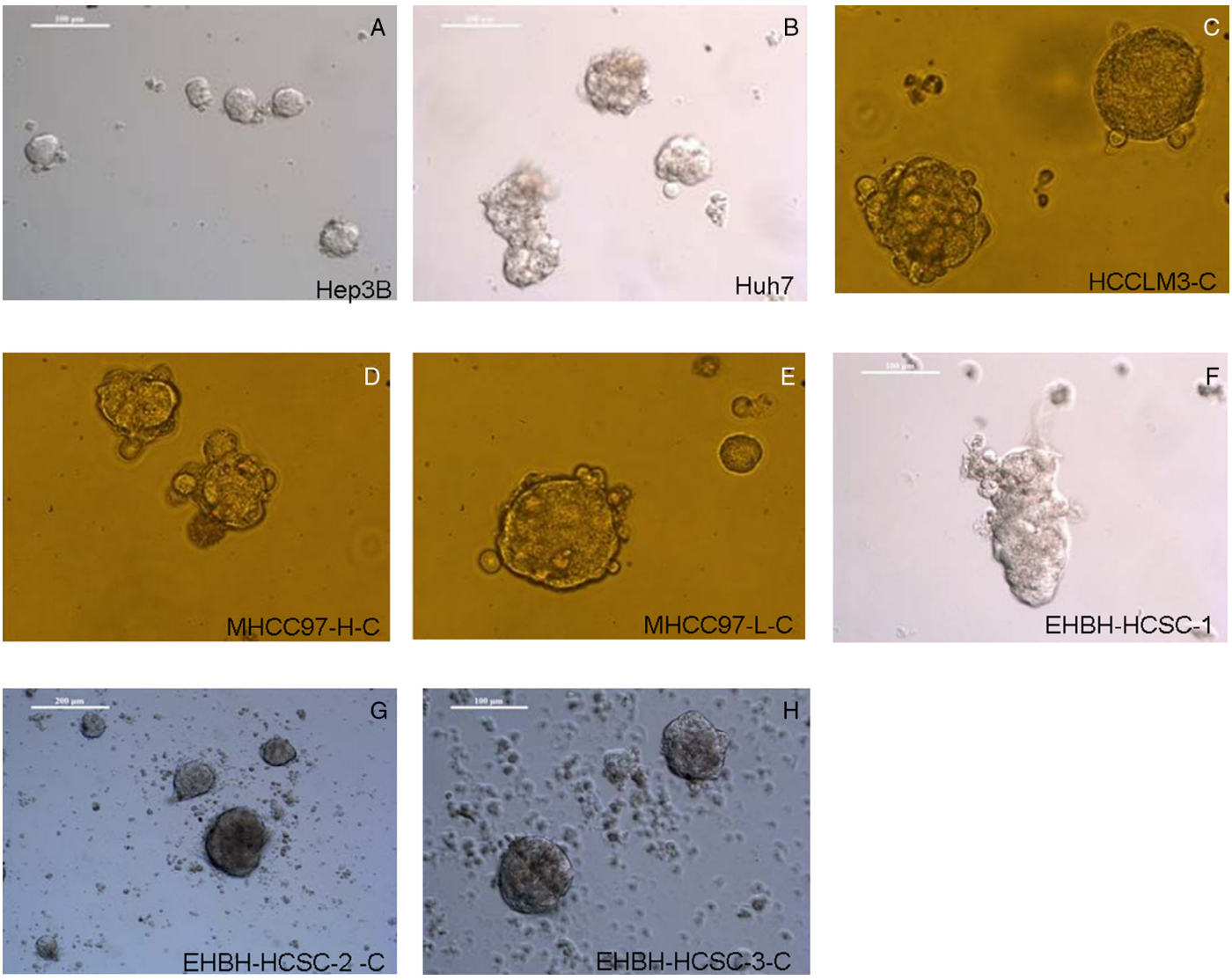

Fig. 1. Cells from 8 HCC cell lines formed the anchorage-independent, self-renewing spheres in C3 medium. Hepatoma cell lines Hep3B, Huh7, HCCLM3, MHCC97-H, (E) MHCC97-L, EHBH-HCSC-1, EHBH-HCSC-2 and EHBH-HCSC-3 could form the anchorage-independent 3-D spheres in C3 medium for 2–3 months (200×). "-C" indicates sphere-forming cells.

cancer metastasis [6]. Herein, we used *in vitro* transwell assay to compare the invasive potentials of sphere-forming cancer cells from Huh7-C and Hep3B with that of their parental cells Huh7 and Hep3B, respectively. The results of the invasion assay showed that more Huh7-C (Fig. 4A, right panel) and Hep3B-C (Fig. 4B, right panel) cells passed through the matrigel than their parental Huh7 (Fig. 4A, left panel) and Hep3B cells (Fig. 4B, left panel), respectively. Quantitative analysis demonstrated that the differences between the number of invasive cells in Huh7-C (Fig. 4C) and Hep3B-C (Fig. 4D) and their parental cells were significant in statistics, indicating that the sphere-forming cancer cells possess more invasive potential than their parental cells *in vitro*.

Cell cycle and tumorigenic capacity of sphere-forming cancer cells from HCC cell lines

Studies have indicated that some CSCs may switch between quiescent, slow-cycling status and active status, similar to the behavior of many types of adult stem cells, and long-term quiescent/dormant pools of human CSCs are present in cancer cells [20,21]. In line with these previous findings, we also found that 74.09% of Hep3B-C and 88.15% of HuH7-C cells were in the G0–G1 phase, while 57.67% of parental Hep3B and 62.67% of parental HuH7 cells were in the G0–G1 phase.

The tumorigenic potentials of the sphere-forming cells from four HCC cell lines were accessed *in vivo* in immunodeficient mice and were compared with that of their parental cells. In the present study, tumorigenicity is defined as the capacity of a certain type of cell with serial dilutions to form measurable xenograft tumor nodules in immunodeficient mice within 3 months. Tumor nodules appeared 3 month after inoculation of 1×10^5 sphere-forming cells derived from all four HCC cell lines, while the same number of HuH7 parental cells failed to form tumor nodules (Table 1), 1000 of Huh7 sphere-forming cells successfully formed tumor nodules in 1 out of 5 mice. Once the cell number was decreased to 100, all parental cells of HCCLM3, MHCC97L and MHCC97H failed to generate any tumor nodules, but their corresponding sphere-forming cells were still capable to initiate the formation of tumor nodules in nude mice with various positive incidences (Table 1). This result clearly demonstrates that the sphere-forming cancer cells possess much stronger tumorigenic potentials than their parental cells, indicating increased stemness.

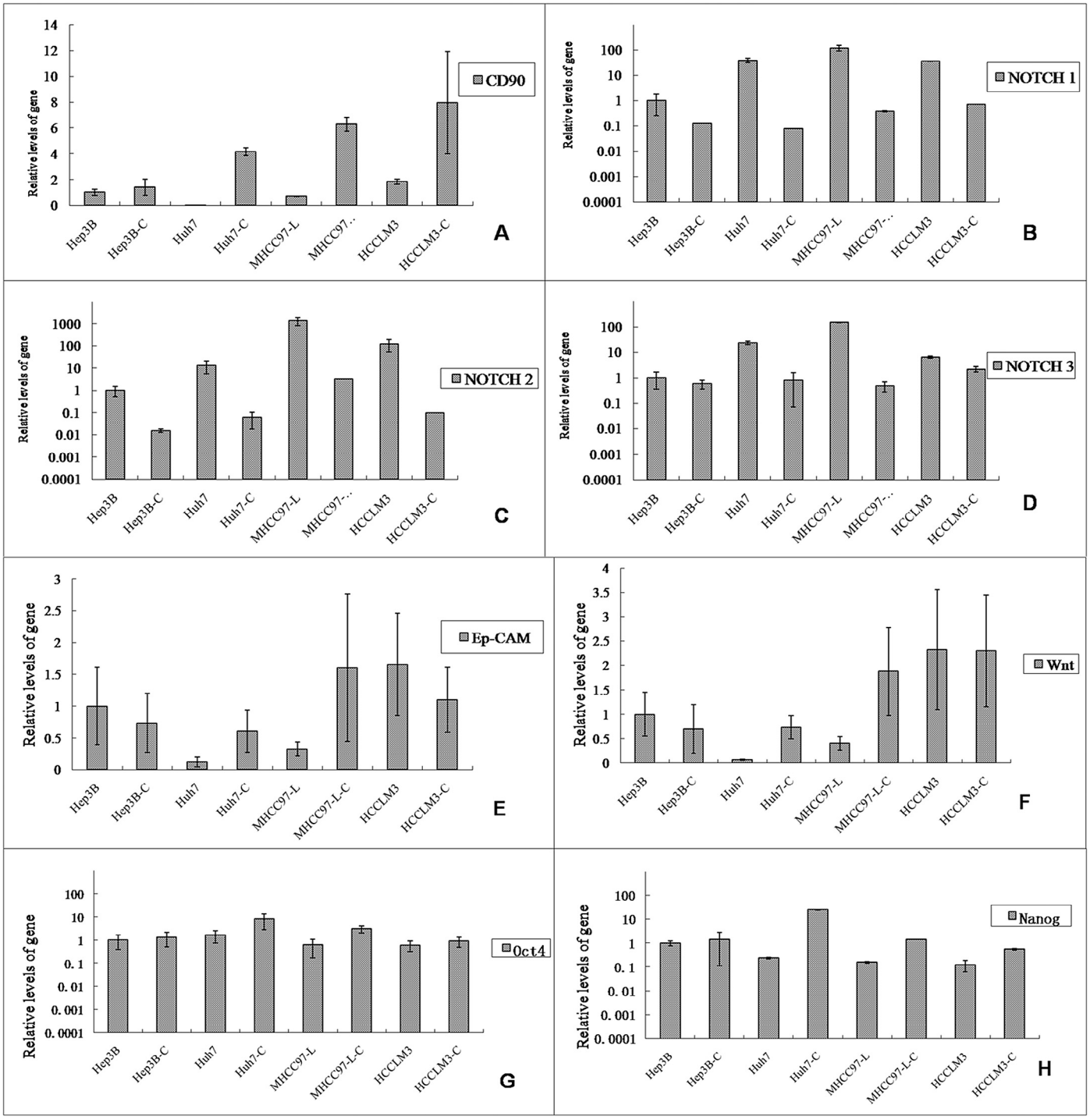

Fig. 2. Expression of CSC-related genes in sphere-forming cells. qRT-PCR analysis showed that in comparison with their parental cells, sphere-forming cells Hep3B-C, Huh7-C, MHCC97-L-C and HCCLM3-C showed differential expression of (A) CD90, (B) NOTCH1, (C) NOTCH2, (D) NOTCH3, (E) Ep-CAM, (F) Wnt-1, (G) Oct4 and (H) Nanog. All experiments were repeated 3 times. Data are the mean ± SD. * $P < 0.05$; ** $P < 0.01$, compared with parent cells.

Sphere-forming tumor cells are more resistant to doxorubicin

Accumulating pieces of evidence have suggested that CSCs are commonly resistant to conventional chemotherapy and contribute to cancer recurrence [22]. To examine whether the self-renewing sphere-forming cells have increased chemoresistance, the sensitivity of the parental Huh7 cells and the tertiary sphere-forming cells toward doxorubicin and sorafenib was assessed. Compared with their parental HuH7 cells, the sphere-forming cells were much more resistant to doxorubicin (Fig. 5A) and sorafenib (Fig. 5B), as their IC50 values were significantly higher than that of their parental Huh7 cells ($p < 0.05$). These results are in agreement with the commonly accepted concept that CSCs are chemoresistant and suggested the significant enrichment of stem cell-like cancer cell populations.

We also analyzed the Doxorubicin-infiltrated cells in both Hep3B and HuH7 cancers by flow cytometry. The results showed that after exposure to 100 μM doxorubicin for 4 hours, the percentage of cells sensitive to doxorubicin decreased after the enrichment, from 84.1%

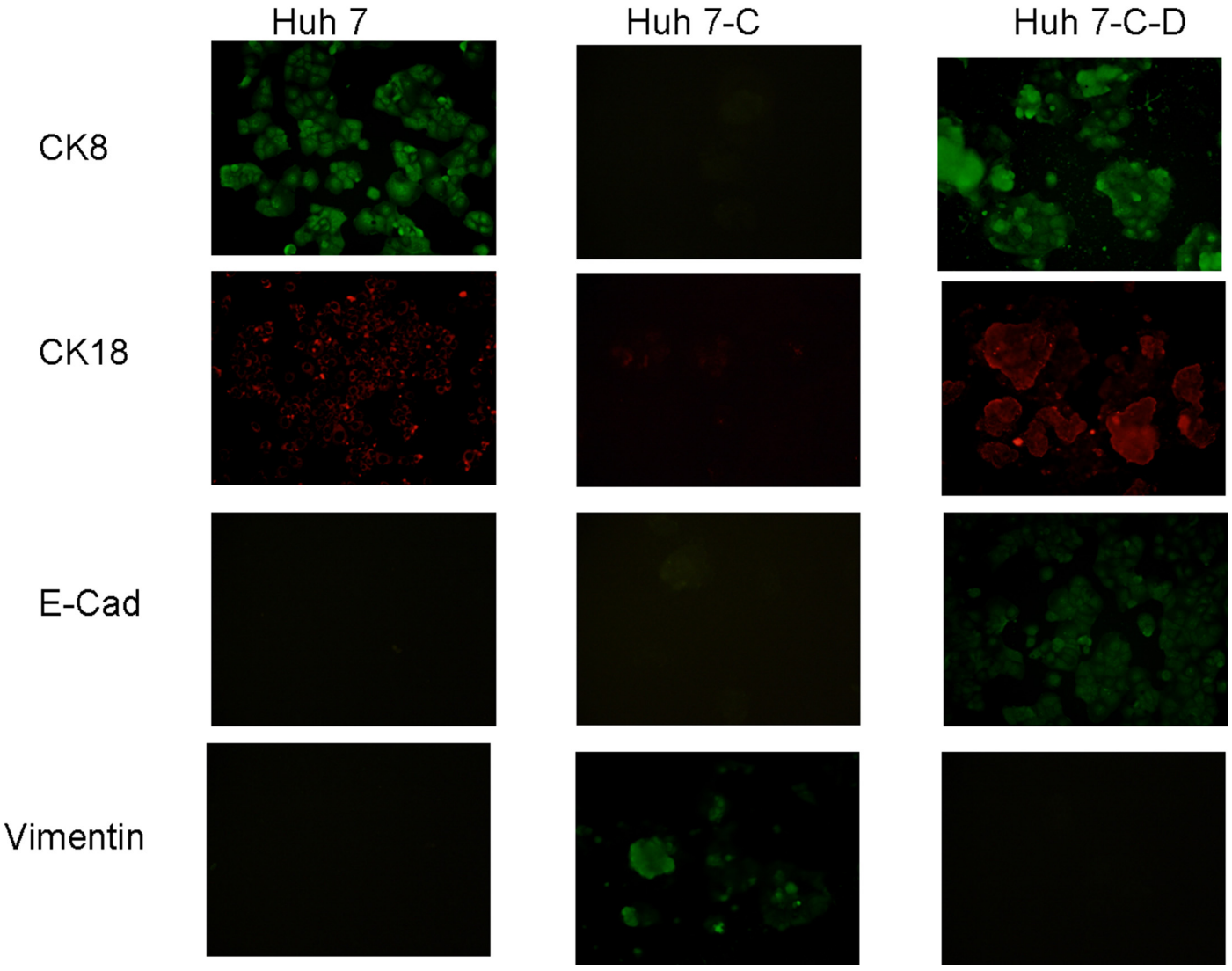

Fig. 3. Immunofluorescence staining of epithelial and EMT markers on sphere-forming cell HuH7-C. Immunofluorescence staining showed decreased expression of CK8/18 and E-cadherin, and elevated expression of Vimentin in sphere-forming cells Huh7-C and in C3 medium, and the changes were reversed after the cells were cultured in serum-supplemented medium. "-C" indicates sphere-forming cell, and "-C-D" is for sphere-forming cells that were cultured in the medium with serum.

(Fig. 5C) in parental Huh7 cells down to 34.5% (Fig. 5D) in corresponding sphere-forming cancer cells, and from 91.3% (Fig. 5E) in parental Hep3B cells down to 77.6% (Fig. 5F) in the sphere-forming cancer cells. This result suggests that the intake of the drugs by the sphere-forming cancer cells is less than that of their parental cancer cells at the same drug concentration, and may partially explain the high level drug resistance in HCSCs.

RNAseq analysis of differential expression of genes and miRNAs in sphere-forming cells

To further verify the enrichment of stem-like cancer cells, RNAseq was used to profile genes and miRNAs differentially expressed in sphere-forming cells and their parental Hep3B, Huh7 and MHCC97-H cells. We identified 406 differentially expressed genes overlapped among groups via fold change method (Table S3). It was found that 90 genes were up-regulated and 316 genes were down-regulated. Heat map shows the clustering of all the significant genes (Fig. 6A). In order to validate the microarray results, we selected several highly differentially expressed genes in Table S3 for validation by RT-PCR. The expression patterns of 9 genes were confirmed by RT-PCR and are shown in Fig. 6B. In order to identify the functional classification of these 406 genes, we performed functional enrichment analysis in the GeneOntology database. The analysis showed that dys-regulated genes in stem-like cancer cells were associated with biological signaling pathways such as the TGF-beta pathway, cell adhesion (PTPRM), PPAR signaling pathway (FADS2), biosynthesis of un-saturated fatty acids (FASN).

During the last decade, it has become clearer that alterations in the expression of several miRNAs are extensively associated with the stemness of CSCs of many human cancers including HCC [23]. Herein, we also revealed the differential expression of miRNAs in sphere-forming cancer cells of Hep3B and Huh7 in comparison with their parental cells. Heat map shows the clustering of all the significant genes (Fig. 6C). We identified, through RNAseq, 18 differentially expressed miRNAs in two sphere-forming cancer cell lines compared with their parental cells. Of them, 9 miRNAs were found up-regulated including miR-450b-5p, miR-478d, miR-375, miR-215, miR-378, miR-760, miR-1296b and miR-1269; and the other 9 miRNAs were downregulated including miR-210, miR-15b*, miR-100, miR-181c, miR-29c, miR-149, miR-199a-3p, miR-199b-3p and miR-22, (Table S4). In order to validate the RNAseq

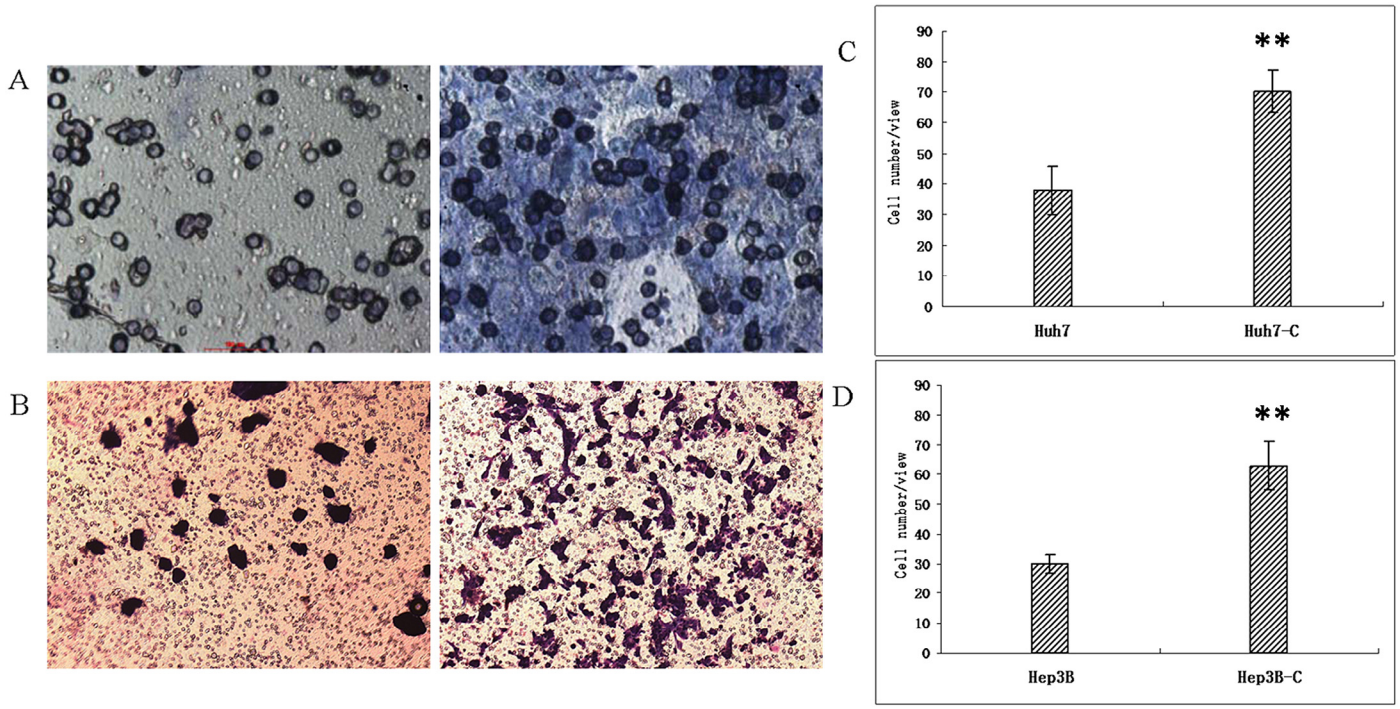

Fig. 4. Enhanced invasive potential in sphere-forming cells Huh7-C and Hep3B-C. Representative photomicrographs showed sphere-forming cells (right panel) of (A) Huh7-C and (B) Hep3B-C that have invaded through matrigel compared with their parent cells (left panel). Quantitative analysis of invaded cells from sphere-forming cells (C) Huh7-C and (D) Hep3B-C was presented as mean values graphed for indicated cells (n = 3 replicates per cell type). Error bars show SD (**$p < 0.01$, compared with parental cells).

results, we selected several miRNAs that were highly differentially expressed in Table S3 for validation by RT-PCR. The differential expressions of the 8 selected miRNAs were all confirmed by RT-PCR (Fig. 6D). Furthermore, we also identified miR-15b to be significantly downregulated, and miR-215 to be significantly up-regulated in two HCC sphere-forming cancer cells. Interestingly, a recent study has identified miR-15b that inhibits CSC growth to be downregulated in breast CSCs [24]. Another study further demonstrated that miR-15b regulated chemotherapy-induced EMT in human tongue cancer cells by targeting BMI1 [25]. Studies also demonstrated that miRNA-215 enhances the chemoresistance of CD133 high-CD44 high colon cancer cells to methotrexate and tomudex by inhibiting cell proliferation and inducing G2-arrest through the suppression of DTL expression [26].

Discussion

The TIC model of HCC formation has gained popularity recently because it provides the explanation to HCC resistance to radiation and chemotherapy, which is a major problem in HCC treatment. Based on this model, the resistance of HCC to radiochemotherapy is due to the existence of a subpopulation of the HCC cells that possess the stem cell characteristics, but are still capable of causing the heterogeneous lineages of cancer cells that constitute the tumor. Despite the evidence supporting the existence of such a subpopulation of stem-like cancer cells in various cancers, due to the lack of clearly demonstrated isolation of CSCs, controversies remain about the prospects of application of the TIC model in cancer therapy.

To better understand the formation of HCC in order to find effective approaches to treat liver cancer, there is a clear need to establish an *in vitro* model that allows the culture and expansion of the stem-like cancer cells. Various methods have been employed in an attempt to isolate and enrich the HCSCs. Immunochemical selection based on the cell surface biomarkers is one of the most commonly used methods. These biomarkers include CD133, CD90 and EpCAM. However, the definitive markers for HCSC selection have yet been identified as it has been found that not all these markers are enriched on the surfaces of the same subset of cells. The difference of dye efflux properties in cancer cells is also employed to distinguish HCSCs from differentiated HCC cells. But at least in the case of Hoechst 33342, concerns arose as the enrichment of SP cells may be a result of Hoechst 33342 toxicity to the non-SP cells whose growth was suppressed by the drug. Physical properties of the HCC cells such as cell density were also applied in TIC selection [8], but the method does not allow the expansion and enrichment of the HCSC subpopulation.

Table 1
Comparison of tumorigenic capacity of parental and sphere-forming cells from different HCC cell lines.

Cell lines	MHCC97-H	MHCC97-H-C	MHCC97-L	MHCC97-L-C	HCCLM3	HCCLM3-C	Huh7	Huh7-C
10^5	5/5	5/5	3/5	5/5	4/5	5/5	0/5	5/5
10^4	0/5	4/5	1/5	5/5	2/5	5/5	0/5	3/5
10^3	1/5	3/5	1/5	5/5	1/5	0/5	0/5	1/5
10^2		1/5		5/5		2/5		0/5

Tumor nodules appeared 3 months after inoculation of 1×10^5 sphere-forming cells derived from all four HCC cell lines, while the same number of HuH7 parental cells failed to form tumor nodules (Table 1), 1000 of Huh7 sphere-forming cells successfully formed tumor nodules in 1 out of 5 mice. Once the cell number was decreased to 100, all parental cells of HCCLM3, MHCC97L and MHCC97H failed to generate any tumor nodules, but their corresponding sphere-forming cells were still capable to initiate the formation of tumor nodules in nude mice with various positive incidence.

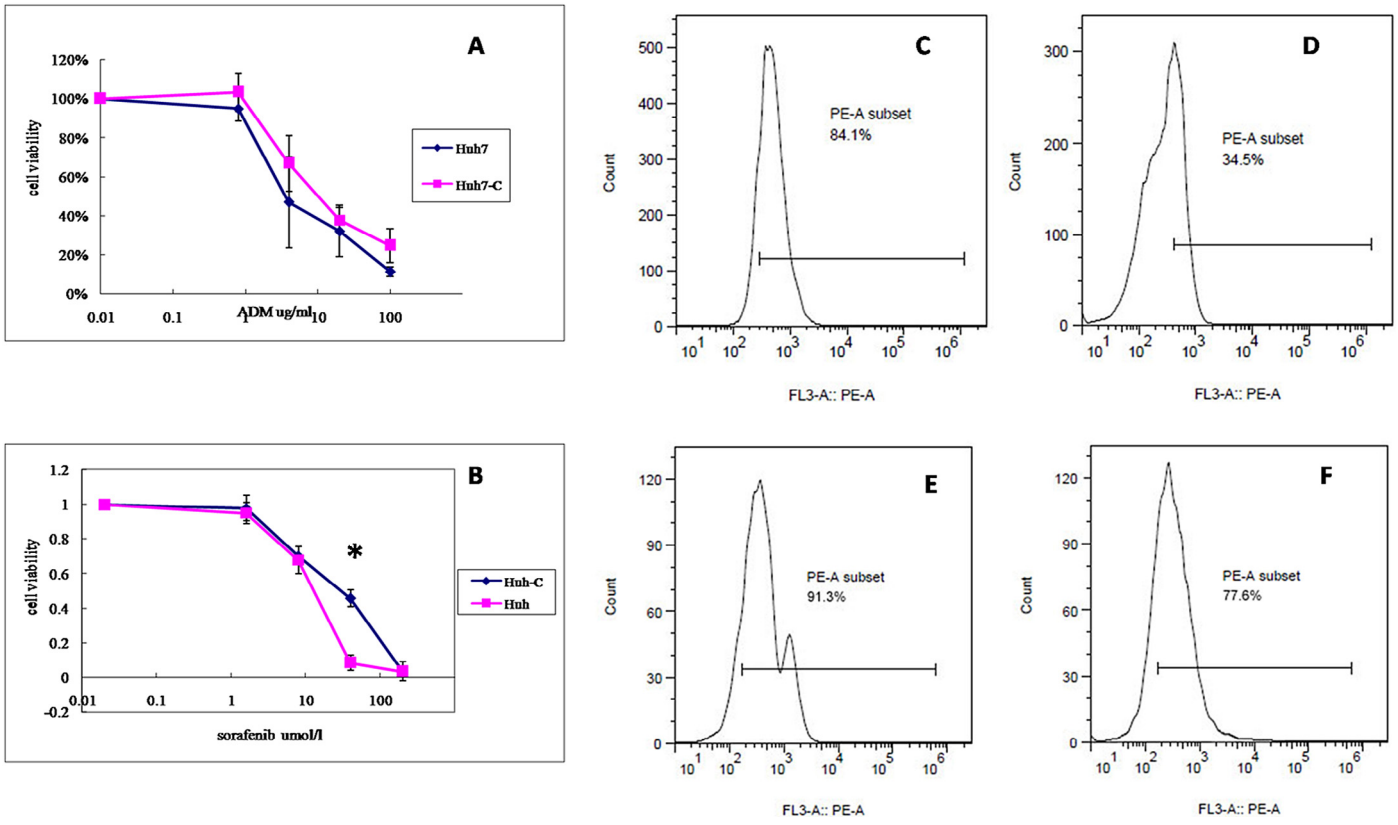

Fig. 5. Resistance of the sphere-forming cells to anti-cancer drugs. (A and B) Compared with parental HuH7 cells, the sphere-forming cells were much more resistant to doxorubicin (A) and sorafenib (B), and their IC50 values were significantly higher than those of the parental Huh7 cells. Data were presented as mean ± SD of three independent experiments in duplicates ($^{*}P < 0.05$ and $^{**}p < 0.01$, compared with parental cells). Subpopulation of sphere-forming cells and parent cells of (C and D) Huh7 and (E and F) Hep3B positive for doxorubicin after being cultured with 100 μm doxorubicin for 4 h, and then measured by FACS.

The approach that uses defined serum-free media has been a powerful method to select and enrich HCSCs, and has many advantages over other methods. The approach defines the *in vitro* conditions that allow the predictable selection and expansion of HCSCs without pre-selection, thus eliminating the problem associated with the various expression patterns of cell surface markers found on different subpopulations of HCSCs. Furthermore, strong evidence has suggested that the microenvironment plays an important role in the initiation and progression of cancer [27,28]. The defined serum-free system is therefore especially useful in understanding the role of the HCSCs in the formation of HCC, and development of approaches that may be more effective in HCC treatment and reduction of the post therapy recurrence of cancer. The defined serum-free media also allow expansion of the HCSCs, and the expanded cells can be used as screening tools for research and drug discovery [29].

In this study, after extensive screening, we have developed a defined serum-free medium and demonstrated that it is effective in HCSC expansion and enrichment. This medium contains DMEM/F12, Neurobasal-A medium, B27 without VA, EGF, the cytokines FGF-10 and IGF-1 and was named as C3 medium. The sphere-forming cancer cells from both HCC cell lines and human primary HCC could be enriched and expanded in the medium. Analyses of stem cell biomarker mRNAs showed that in comparison with their parental cells, the sphere-forming cancer cells cultured in this medium have higher levels of CD90 expression, and decreased expressions of NOTCH1, 2 and 3, as well as Wint-1 and EpCAM, indicating the enrichment of cancer cells with stem cell characteristics (stem-like cancer cells). Recent studies indicate that CSC is a result of dedifferentiation from cancer cells, a process resembling EMT; we also examined some EMT markers on these sphere-forming cancer cells, and found that the expression of cytokeratins 8/18 and E-cadherin decreased dramatically, and vimentin was up-regulated, indicating the gain of mesenchymal cell markers. Moreover, *in vitro* transwell assay showed that the sphere-forming cancer cells possess more invasive potential than their parental cells, a result in accordance with the observation that some CSCs promote metastasis [6]. As resistance to chemotherapeutic agents is a widely accepted characteristic of CSCs [22], we also examined the resistance of these sphere-forming cancer cells to doxorubicin and sorafenib. As demonstrated by the IC50s, the sphere-forming cancer cells are more resistant to both drugs. Since the ultimate criterion in verifying the stemness of cancer cells is their tumor-initiating ability [30], we also analyzed the abilities of these sphere-forming cells to initiate tumor in immunodeficient mice. It was found that these sphere-forming cancer cells have much higher capacity in the initiation of xenograft tumor nodules than that of their parental cells, with as low as 100 cells being able to initiate the formation of the tumor nodules. This result strongly suggested that the enriched sphere-forming cells are indeed HCSCs.

We also analyzed the gene expression pattern in both the sphere-forming cancer cells and their parental cells. Results showed that 406 genes were expressed differentially, with 90 genes up-regulated and the rest 316 genes down-regulated. Further analysis revealed that the down-regulated genes are associated with pathways such as the TGF-beta pathway, cell adhesion, PPAR signaling pathway, and biosynthesis of unsaturated fatty acids. The significance of the differential gene expression profile is subject to future study. Recent studies have also shown that miRNAs play an important role in CSCs [23]. To gain an insight into the regulation of the enrichment of the

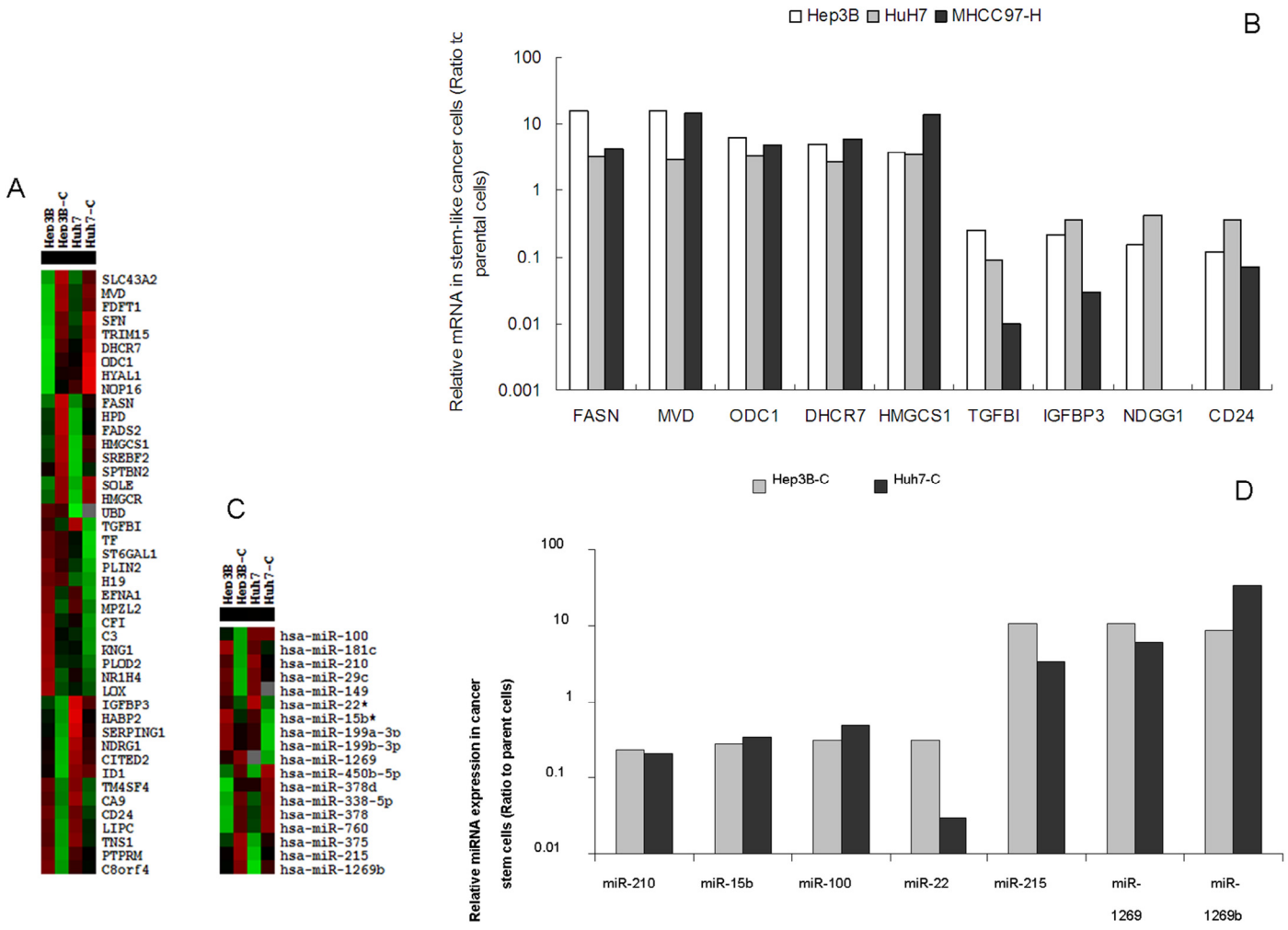

Fig. 6. Genes differentially expressed in the sphere-forming cells and their parental cells. (A) The heat map shows the clustering of all the significant genes (22 genes) by RNAseq analysis. (B) Nine selected genes that were differentially expressed were validated by qRT-PCR. The results were consistent with the data of RNAseq. (C) The heat map shows the clustering of all the significant miRNA (18 miRNA) by RNAseq analysis. (D) Differentially expressed miRNAs were identified by RNAseq and validated by qRT-PCR. The mRNA expression of each gene or miRNA was presented as the ratio to reference gene and small RNA. Data were presented as mean ± SD of three independent experiments in triplicates. * $P < 0.05$; ** $P < 0.01$, compared with parental cells.

HCSCs, we compared the profiles of the expression of miRNAs in the sphere-forming cancer cells with those of their parental cells. Analysis showed the up-regulation of 9 miRNAs and down-regulation of another 9 miRNAs. One of down-regulated miRNAs is miR-15b which has been shown to inhibit the growth of CSCs and is also down-regulated in breast CSCs [24].

To better understand the etiology of HCC and develop effective anti-cancer approaches, there is a clear need of *in vitro* model systems for the study of the HCSCs. In the general CSC field, cancer stem cells have been established by using markers or the unique physical and chemical properties of stem cells to separate subpopulations from existing tumor lines. In our study, CSCs were selected from tumors or tumor cell lines using defined serum-free conditions. In this medium, we gained a group of cells which showed typical stem cell properties. However, whether liver cancer stem cells are just one kind of cell or a group of cells, do they have very defined surface markers? Are cancer stem cells in a relatively stable state or in a process of changing? All of these questions need to be explored with continuous efforts. Fortunately, we are getting more and more closer to the goal.

Taken together, we have successfully developed a medium that has enabled us to effectively enrich the population of sphere-forming cancer cells from both hepatoma and human primary HCC cells. These cells exhibited typical stem cell properties, such as enhanced stem cell markers, gain of EMT properties and drug resistance, and more importantly, stronger tumor-initiating capabilities. Targeting HCSCs may bring hope in curing HCC, but the progress on HCSC research has been slow largely due to the difficulties either in identifying these HCSCs in liver cancers or in isolating such HCSCs. Our culture system makes it possible to study HCSCs *in vitro*, which may contribute to the development of novel cell therapies and new drugs for the treatment of HCC.

Conclusions

We established a novel culture system that enlarged these hepatic stem-like cancer cells from both hepatoma cells and human primary HCC cells. These cells showed typical stem cell properties, such as enhanced stem cell markers, gain of EMT properties and drug resistance, and more importantly, stronger tumor-initiating capabilities. The culture system may help to establish an *in vitro* model for hepatic cancer stem cell (HCSC) studies, which may contribute to the development of novel cell therapies and new drugs for the treatment of HCC.

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Conflict of interest

The authors declare that they have no competing interests.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.canlet.2015.02.006.

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