Original Article

Establishment of NOD/SCID mouse models of human hepatocellular carcinoma via subcutaneous transplantation of histologically intact tumor tissue

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Abstract: Hepatocellular carcinoma (HCC) is one of the most deadly human cancers, but it is very difficult to establish an animal model by using surgical specimens. In the present experiment, histologically intact fresh surgical specimens of HCC were subcutaneously transplanted in non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice. The biological characteristics of the original and the corresponding transplanted tumors and cell lines were investigated. The results showed that 5 new animal models and 2 primary cell lines were successfully established from surgical specimens. Hematoxylin-eosin staining showed that xenografts retained major histological features of the original surgical specimens. The two new cell lines had been cultivated for 3 years and successively passaged for more than 100 passages *in vitro*. The morphological characteristics and biologic features of the two cell lines were genetically similar to the original tumor. The subcutaneous transplant animal models with histologically intact tumor tissue and primary cell lines could be useful for *in vivo* and *in vitro* testing of anti-cancer drugs and be ideal models to study various biologic features of HCC.

Key Words: Animal model; hepatocellular carcinoma; subcutaneous transplantation; surgical specimens

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Introduction

Liver cancer is one of the most common malignancies in the world and the third most common cause of cancer-related death (1,2). The highest age-standardized mortality rate was reported for China (34.7/100,000), which alone accounts for 53% of all liver cancer-related deaths worldwide (3). The incidence of hepatocellular carcinoma (HCC) is slowly but steadily increasing, particularly in the Chinese population, owing to endemic hepatitis B virus (HBV) infection, and in the West, primarily due to increased hepatitis C virus (HCV) infection (4-6). Liver resection and liver transplantation are curative treatments for early-stage HCC. However, HCC is usually diagnosed at an advanced stage accompanied by recurrence and metastasis. Although progress has been made in clinical treatment, overall survival for patients with HCC remains extremely poor (7). Therefore, studies on the mechanism, angiogenesis, progression, recurrence, and metastasis of HCC are of great importance.

Animal models are important tools for investigating the pathogenesis of diseases and developing treatment strategies (8,9). Numerous experimental models have been developed to mimic the characteristics of different stages of HCC in humans and test novel drug candidates (10-13). In the past three decades, a great number of human HCC cell lines have been established (14). In 1963, Chen *et al.* established the first human HCC cell line, BEL-16. Alexander *et al.* established the famous human HCC cell line PLC/PRF/5. Dong *et al.* established the human HCC cell line SMMC-7721 in 1977, and this cell line remains one of the most commonly used human HCC cell lines in China. In 1996,

Leveille-Webster *et al.* established intrahepatic xenografts of human HCC in severe combined immunodeficiency (SCID) mice to study multidrug resistance (15). However, these cell lines and animal models are unable to meet the needs of clinical basic research of HCC, which has very complex etiology such as HBV or HCV infection, aflatoxin B1 (AFB1) exposure and high genetic heterogenicities.

In the present experiment, histologically intact fresh surgical HCC specimens were subcutaneously transplanted into non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice, which were combined T cell, B cell, and NK cell deficiency. The biological characteristics of the original and corresponding transplanted tumors and cell lines were investigated.

Materials and methods

Mice

Six- to eight-week-old NOD/SCID male and female mice and T cell-immunodeficient BALB/c-nu/nu mice were maintained under standard conditions according to our institution's guidelines. All animal experiment protocols used in this study were approved by the Shanghai Medical Experimental Animal Care Commission at Shanghai Jiaotong University (approval ID ShCI-12-023).

Surgical human HCC specimens

Fresh surgical specimens were obtained with informed written consent from 24 patients with HCC who had undergone liver cancer resection at the Liver Cancer Institute of Zhongshan Hospital, Fudan University (Shanghai, China). The specimens were rinsed, preserved in ice-cold serum-free Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, USA), and sent to our laboratory within 2 h.

Subcutaneous transplantation of surgical specimens

After the necrotic and non-cancerous tissues of the specimens were removed, the remaining cancerous tissues were cut into small pieces of approximately 2 mm^3 in size, and four or five pieces of tissue were transplanted subcutaneously into the right flanks of NOD/SCID mice. Depending on the surgical specimen's size, tumor pieces from each patient were generally transplanted into between two and eight mice. Tumor growth was monitored once per week by palpation. Once the subcutaneous tumor reached 10-15 mm in diameter, it was removed and cut into approximately 2 mm × 2 mm × 2 mm pieces, which were transplanted into NOD/SCID mice using the aforementioned method. When the tumor had been passaged three times, it was transplanted into nude mice, and tumor formation and growth were observed. At the same time, the tumor was used to establish an orthotopic transplantation animal model. The subcutaneous tumor was removed and cut into approximately 2 mm × 2 mm × 2 mm pieces, which were transplanted into the left hepatic lobe of the nude mice. Tumor formation was monitored starting 1 week after transplantation. After 6 weeks, all of the mice were sacrificed, and the tumor masses and murine liver tissue samples were dissected by Hematoxylin-eosin (H&E) staining. If a surgical specimen had not grown in NOD/SCID mice after 3 months, it was considered an unsuccessful transplantation.

Primary cell lines and cell culture

The xenografts were removed and used for primary culture *in vitro*. Primary cell lines of HCC were established by directly cutting tumor fragments into small pieces using microscissors and placed orderly these small pieces on culture dishes. After 4-6 h, DMEM/F12 (Sigma-Aldrich, USA) containing 10% heat-inactivated fetal bovine serum (FBS; Biowest, France), supplemented with 100 IU/mL penicillin G and 100 μg/mL streptomycin (Sigma-Aldrich, USA), were added to the dishes. The medium was routinely changed every 2-3 d. The primary cell cultures were maintained at 37 °C in a 5% CO_2 and 95% humidified atmosphere. The cell lines were continuously passaged until mesenchymal cells were no longer apparent. Cell morphology was viewed under light microscope and representative pictures were taken.

Immunocytochemistry

Primary cells were directly cultured on slides. Alpha-fetoprotein (AFP), albumin, cytokeratin 18 (CK18), cytokeratin 19 (CK19), Hep Par 1, fibroblast, Oct4, Notch1, CD133, DLK1 and CD44 were detected by immunocytochemistry using a two-step labeled avidin-biotin immunoperoxidase method, as recommended by the protocol. For the primary antibody dilutions, the concentrations were employed according to *Table 1*. The results were visualized and photographed using an Axioskop 2 microscope (Carl Zeiss, Germany) with a DP70 CCD

Table 1 Antibodies used in experiments

Antibody	Company	Cat No.	Dilution
AFP	Beckman Coulter	IM1595	Ready to use
Albumin	DAKO	A0001	1:100
CK18	Santa Cruz	SC-8020	1:20
CK19	Santa Cruz	SC-6278	1:25
Hep Par 1	DAKO	M7158	1:10
Fibroblast	DAKO	M0877	1:25
Oct4	Santa Cruz	SC-5279	1:10
Notch1	Beckman Coulter	552466	1:25
CD133	Santa Cruz	SC-19365	1:25
CD90	BD	555593	1:10
DLK1	Abcam	ab21682	1:25
CD44	Santa Cruz	SC-7297	1:10

AFP, alpha-fetoprotein; CK18, cytokeratin 18; CK19, cytokeratin 19

system (Olympus, Japan).

Cell growth curves

Approximately 1×10^4 cells were added into each well of a 96-well plate. Cell numbers in three wells were counted in a hemocytometer every 24 h for 7 consecutive days, and cell growth curves were plotted.

Migration and invasion assays

Cell migration and invasion assays were measured by using 6.5-mm Transwell chambers (8 μm pore size; Corning). For invasion assays, 34 μg of Matrigel (BD Biosciences, catalogue No. 354234, MA, USA) was coated onto each filter and the chamber was incubated at 37 °C for 2 h to produce the artificial basement membrane. In both assays, tumor cells in serum-free DMEM (200 μL containing 1×10^5 cells) were added into the upper compartment of the chamber, and 800 μL of conditioned medium was added to the lower compartment. Plates were incubated at 37 °C in 5% CO_2 for 12 h and 24 h for migration assays or 24 h and 36 h for invasion assays. The non-migratory or non-invasive cells were wiped from the upper chambers with cotton wool. The undersides of the chamber were fixed in 100% methanol for 30 min, air-dried, stained with 0.1% crystal violet, and photographed with a CKX41 microscope (Olympus, Japan). Ten random fields were analyzed for each Transwell insert, and the number of cells that had migrated was counted. Assays were conducted for three independent times.

In vivo growth of HCC primary cells

Exponentially growing cells were harvested with trypsin-EDTA and resuspended in FBS-free DMEM. For subcutaneous (s.c.) inoculation, approximately 2×10^6 cells in 0.2 mL culture medium were injected s.c. into the right flanks of nude mice (n=8), tumor growth was monitored twice per week, and tumor diameters were measured using vernier calipers. The tumor volume was calculated on the basis of the assumption that the tumors were ellipsoid. All eight mice were killed after 4 weeks, and visible tumors were fixed in 10% buffered formalin and processed for H&E staining. Any metastasis to the lungs, liver, kidneys, spleen, or lymph nodes was noted.

Flow cytometry

Exponentially growing cells were harvested, and single-cell suspension containing 1×10^6 cells was prepared. The cells were treated following a standardized protocol. Molecular markers, including CD133, CD90, EpCAM, CD44, and CD24, which are considered to be cancer stem cell markers, were detected by flow cytometry.

Statistical analysis

Statistical analysis was performed using SPSS version 18.0

Table 2 Clinical and pathologic features of patient material established animal models of HCC in NOD/SCID mice

Patient No.	Age/ sex	Pathologic diagnosis	Edmonson grade	Hilar lymph node metastasis	Treatment	Success in s.c. transplantation	Spontaneous regression	Success in establishment of cell line
5	65/male	HCC	II	yes	hepatectomy	yes	no	yes
8	36/male	Mixed HCC	II	no	hepatectomy	yes	no	no
9	41/female	HCC	II	no	hepatectomy	yes	no	no
10	56/male	HCC	II	yes	hepatectomy	yes	no	yes
19	32/female	HCC	II	no	liver transplantation	yes	yes	no

HCC, hepatocellular carcinoma; NOD/SCID, non-obese diabetic/severe combined immunodeficiency; s.c. transplantation: subcutaneous transplantation

Table 3 The *in vivo* growth of subcutaneous transplantation of human HCC patient No. 5 in NOD/SCID mice

No. of passages	No. of animals	No. of tumor formation	Tumor formation rate (%)	Passage of time (d)
1	8	1	12.5	91
2	8	7	87.5	52
3	8	8	100	45

HCC, hepatocellular carcinoma; NOD/SCID, non-obese diabetic/severe combined immunodeficiency

(SPSS Inc. Chicago, IL, USA). In this study, quantitative data were expressed as the $\bar{x}\pm s$. Significant differences between the groups were compared using Student's *t*-test. $P<0.05$ was considered statistically significant.

Results

Establishment of patient-like HCC animal models in NOD/SCID mice

Twenty-four surgical HCC specimens were obtained, from which five human HCC animal models were successfully established in NOD/SCID mice by subcutaneous transplantation of histologically intact tumor tissues. The tumorigenicity rate reached 20.83% (5/24). In the first generation, tumors grew relatively slowly with different latency periods ranging from 1 to 3 months (*Table 2*). However, tumor formation rate was increased and passage of time was gradually shortened with increasing passages (*Table 3*). The growth of these human HCC models was variable, for example, the volume of one tumor was 2,782.66±1,343.99 mm^3 after 7 weeks, whereas that of another tumor reached 7,255.40±2,034.92 mm^3 in 6 weeks. One of the five established tumors spontaneously regressed in the third generation, whereas the other four tumors continued to grow for 10 passages *in vivo*. For orthotopic transplantation, the tumor take rate was 100% (8/8) in nude mice. The resultant tumors closely resembled the original surgical specimens histologically, and they were demonstrated to possess the characteristics of HCC (*Figure 1*).

Establishment of primary HCC cell lines

The established subcutaneous xenograft models were used in an attempt to establish primary human HCC cell lines. One specimen was taken from a 65-year-old man with Edmonson grade II HCC. His serum AFP level was 125.6 ng/mL, and he was positive for HBsAg, HBeAg, and HBeAb. Additionally, the patient exhibited extensive intrahepatic and hilar lymph node metastases, but no distant metastases were noted during surgery. The other specimen was obtained from a 56-year-old man with Edmonson grade II HCC. His serum AFP and carcinoembryonic antigen (CEA) levels were 466.9 and 6.48 ng/mL, respectively, and he was positive for HBsAb and HBeAb. Like the other patient, he exhibited extensive intrahepatic and hilar lymph node metastases, but no distant metastases were noted during surgery.

During the first month of primary culture, proliferating tumor cells grew slowly and formed small colonies, some of which were surrounded by a great number of fibroblasts. However, these fibroblasts gradually disappeared as the

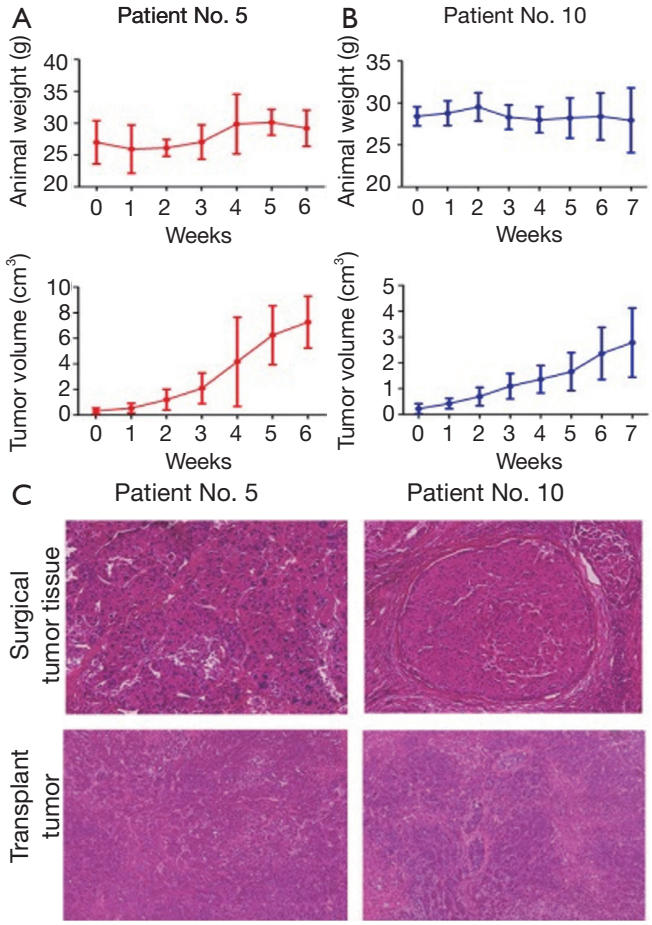

Figure 1 The *in vivo* growth of subcutaneous transplantation of human HCC surgical specimens in NOD/SCID mice. The cancerous tissues were cut into small pieces of approximately 2 mm^3 in size, and four or five pieces of tissue were transplanted subcutaneously into the right flanks of NOD/SCID mice (n=8). Tumor growth was monitored once per week and tumor diameters were measured using vernier calipers. The tumor volume was expressed as $\bar{x}\pm s$. A. The animal weight and tumor volume of human HCC patient No.5; B. The animal weight and tumor volume of HCC patient No.10; C. H&E staining of surgical tumor tissue and their orthotopic transplant tumor. The magnification of photograph was 200×

passage number increased. Two of the four established cell lines could be maintained and continuously cultured *in vitro*. The resultant two cell lines were established in culture and designated HCC-LY5 and HCC-LY10, respectively. The success rate of culturing primary cell lines was thus 50%. At present, the cell lines have grown continuously for 100 passages and discontinuously for more than 3 years *in vitro*.

Identification of human HCC cell lines by immunocytochemistry

Primary cells were directly cultured on slides, and the expression of molecular markers was detected by immunocytochemistry. The results showed that HCC-LY5 and HCC-LY10 cells were positive for the liver cell-associated proteins albumin, CK18, CK19, and Hep Par1; the liver cancer stem cell-associated proteins Oct4, Notch1, CD90, DLK1 and CD44. HCC-LY5 cells, but not HCC-LY10 cells, expressed CD133 and AFP (*Figure 2*).

Biological characteristics of primary cell lines

HCC-LY5 and HCC-LY10 cells grew as monolayer polygons with clear boundaries and exhibited a similar morphology. The two cell types were both characterized by an epithelial-like shape with prominent nuclei. The *in vitro* growth of the two cells was close ($P>0.05$). For the migration assay, the numbers of cells that penetrated the artificial basement membrane were (126.46±18.07) and (219.13±33.75) cells per high power field at 12 and 24 h, respectively, for HCC-LY5, and (0.63±0.68) and (5.53±1.90) cells per high power field, respectively, for HCC-LY10 ($P<0.05$). For the invasion assay, the numbers of cells that penetrated the Matrigel were (39.69±27.85) and (74.93±20.12) cells per high power field at 24 and 36 h, respectively, for HCC-LY5, and (5.27±3.41) and (9.22±4.94) cells per high power field, respectively, for HCC-LY10 ($P<0.05$). Thus, HCC-LY5 cells had higher metastatic potential than HCC-LY10 cells. Primary cultured cells at regular passages were injected subcutaneously into nude mice, and tumor formation was observed. The tumorigenicity of these two primary cell lines reached 100%; the mean latent period was 18 d for both cell lines. The tumor volume was 2,543.84±897.70 mm^3 for HCC-LY5 cells and 2,164.91±864.43 mm^3 for HCC-LY10 cells. No metastases were identified in nude mice bearing s.c. tumors (*Figure 3*).

Detection of HCC molecular markers by flow cytometry

A large number of molecular markers have been demonstrated to be associated with cancer stem cells. In

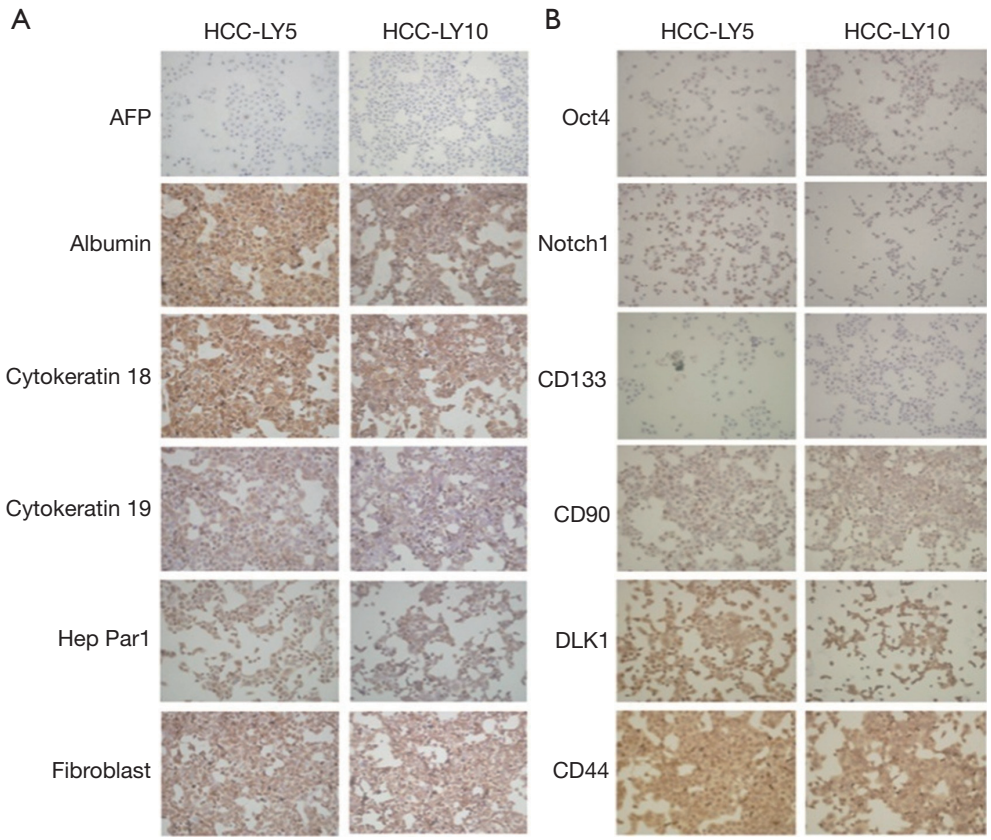

Figure 2 The identification of human HCC cell lines by immunocytochemistry. AFP, Albumin, CK 18, CK 19, Hep Par1, Fibroblast, Oct4, Notch1, CD133, CD90, DLK1 and CD44 were detected by immunocytochemistry, respectively. A. The liver cell associated proteins; B. The liver cancer stem cells associated proteins. The magnification of photographs was 200×

this experiment, several molecular markers were detected by flow cytometry. In total, 0.1%, 0.4%, 0.1% and 0.8% of HCC-LY5 cells were positive for CD133, EpCAM, CD44 and CD24, respectively, and 28.9%, 0.5% and 10.2% of HCC-LY10 cells were positive for EpCAM, CD44 and CD24, respectively (*Table 4*, *Figure 4*).

Discussion

HCC is a prevalent disease worldwide, particularly in China. Patients often die from the recurrence and metastasis of HCC. However, the mechanisms of HCC recurrence and metastasis are poorly understood (16). Liver cancer animal models are important tools for understanding tumor initiation, progression, and metastasis and improving tumor diagnosis, prevention, and treatment. There are four types of HCC models: spontaneous, induced, transplantable, and transgenic/knockout animal models (1,17). Each model has its own advantages and disadvantages. Of these four types, the transplantable animal model is the most widely used. However, the success rate for establishing HCC cell lines is extremely low (16). Compared with other tumor types (18,19), it has been more difficult to establish liver cancer animal models and cell lines. Sun *et al.* established a highly metastatic model of human HCC in nude mice via the orthotopic implantation of histologically preserved metastatic tumor tissues from 30 surgical specimens, but they reported a transplant success rate of only 3.33% (20,21). To further improve the success rate of tumor transplantation, SCID mice can be used as transplant hosts. In previous research, a total of 326 fresh tumor specimens, primarily gastrointestinal and female genital tissue, were engrafted in NOD/SCID mice, and 54 tissue lines were successfully established (22). Human breast carcinoma animal models were established in SCID mice using intact tissue specimens, and the transplantation success rate

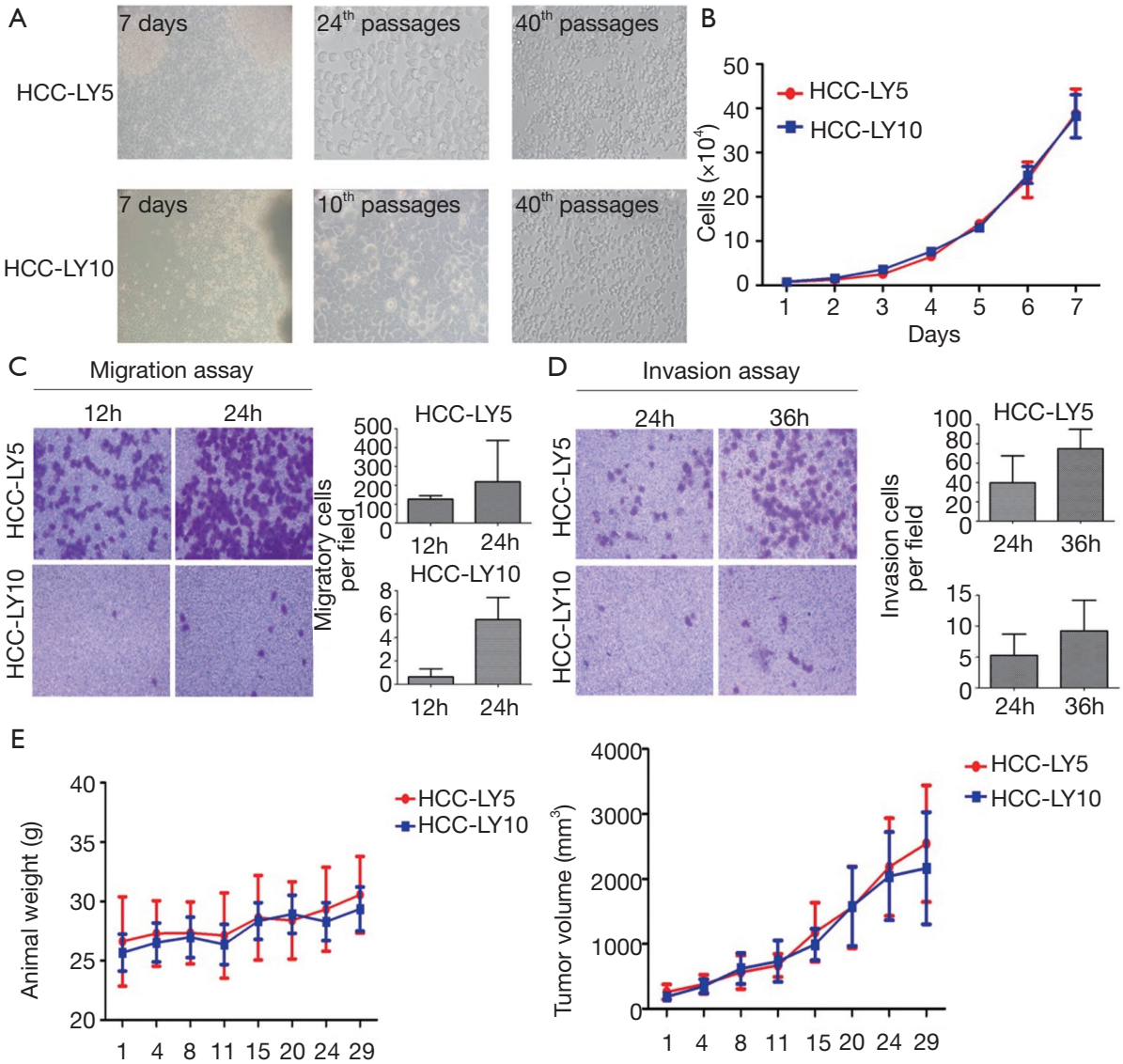

Figure 3 Biological characteristics of the primary cell lines. A. Morphological appearance of each of the established HCC cell lines in culture medium; B. The *in vitro* growth curves of HCC-LY5 and HCC-LY10 cells. 1×10^4 cells were seeded in 96-well plates, and cell proliferation was measured by a hemocytometer every 24 h for 7 consecutive days. The results are expressed as $\bar{x}\pm s$; C. Transwell migration assay in HCC-LY5 and HCC-LY10 cells. 1×10^5 cells were seeded into Transwell plates and cultured for 12 and 24 h at 37 °C; D. Transwell Matrigel invasion assay in HCC-LY5 and HCC-LY10 cells. 1×10^5 cells were seeded into Matrigel-coated Transwell plates and cultured for 24 and 36 h at 37 °C. The cells that had invaded or migrated to the underside of the inserts were then stained with crystal violet. The magnification of photograph was 200×. The results are expressed as the $\bar{x}\pm s$. E. The *in vivo* growth of primary HCC cell line. 2×10^6 cells were injected subcutaneously into the right flank of nude mice (n=8), tumor growth was monitored twice per week, and tumor diameters were measured using vernier calipers. Tumor volume=1/2 (length × width × height)

Table 4 Detection of HCC molecular markers by flow cytometry in HCC-LY5 and HCC-LY10 cells

	CD133 (%)	EpCAM (%)	CD44 (%)	CD24 (%)	CD45 (%)
HCC-LY5	0.1	0.4	0.1	0.8	0
HCC-LY10	0	28.9	0.5	10.2	0

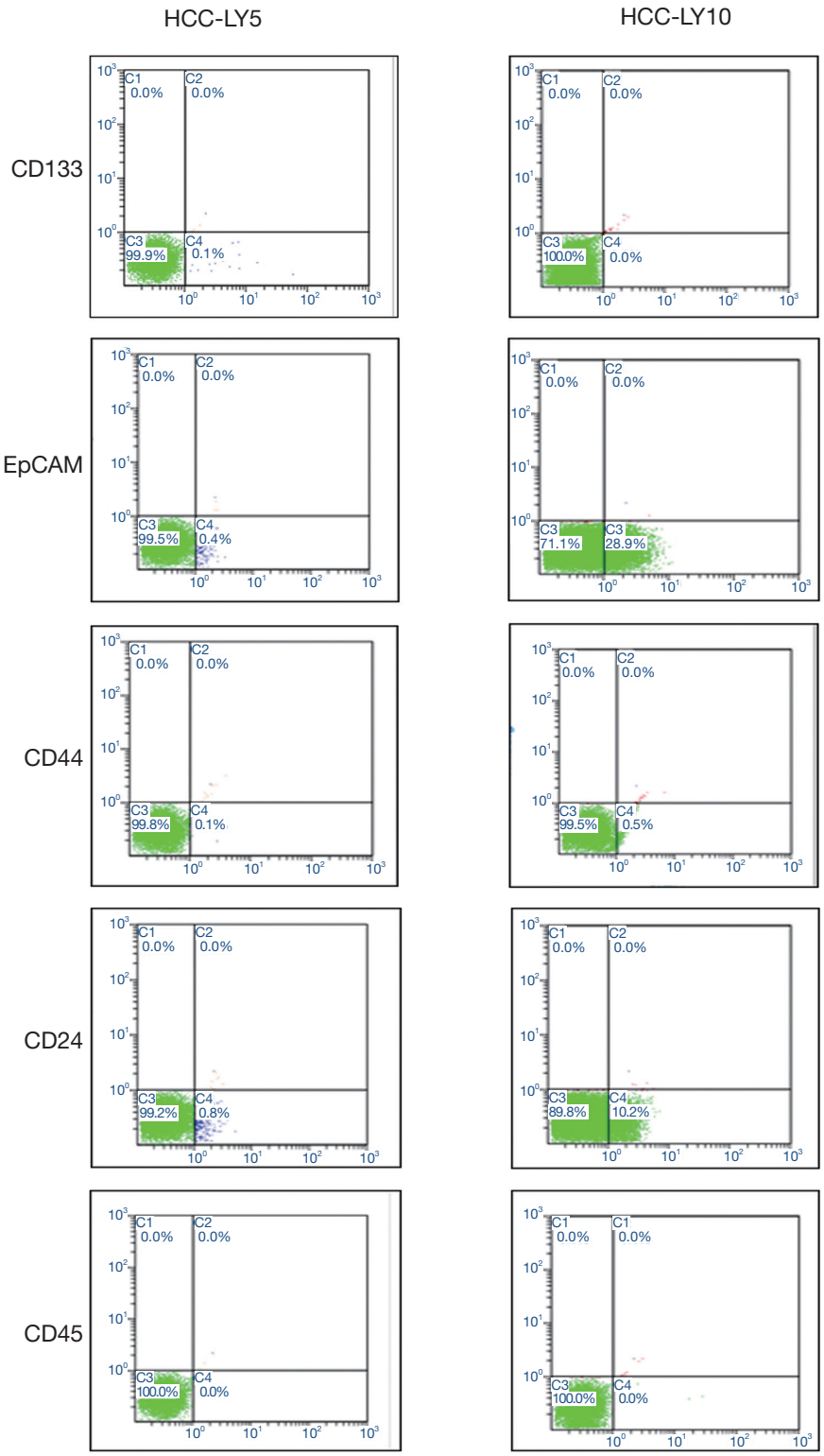

Figure 4 Detection of HCC molecular markers by flow cytometry in HCC-LY5 and HCC-LY10 cells. CD133, EpCAM, CD44 and CD24 were detected by flow cytometry. CD45 was detected as a control

was 50% (23). In the present experiment, histologically intact fresh HCC surgical specimens were subcutaneously transplanted in NOD/SCID mice with a greatly improved transplantation success rate of 20.83% (5/24). The histological characteristics of the resulting tumors were similar to those of the original HCC surgical specimens.

Cancer cells obtained from surgical samples can be maintained in primary culture for only a short time. Krause summarized the factors affecting the success of culture growth (24). The highest cause of failure is a lack of adequate viable cells, which are not likely to thrive *in vitro*. Another complication is the overgrowth of promising tumor cells by fibroblasts. Compared with using surgical samples to directly establish cell lines, a xenograft from an ideal animal model bearing a human tumor would provide more viable cells and a continuous source of live tumor tissue. In the present experiment, we used surgical specimens to directly establish primary HCC cell lines, but no cell lines could be established. However, established subcutaneous xenografts were used in an attempt to establish primary human HCC cell lines. Two of the four established cell lines could be maintained and continuously cultured *in vitro*, resulting in a 50% success rate. The two primary cell lines were grown continuously for 100 passages and discontinuously for more than 3 years *in vitro*.

In summary, *in vivo* and *in vitro* HCC models and cell lines provide us with a useful platform to study tumor growth and metastasis and preclinically screen antitumor therapies.

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