Establishment and characterization of an immortalized human hepatocyte line for the development of bioartificial liver system

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Abstract For further research and application of Bioartificial liver systems (BAL), active proliferation capacity and full hepatic functionality of the biomaterials is mandatory. However, there are still no suitable cell lines meeting the requirements for an ideal cell source in BAL development that makes it necessary to explore other sources. Here, we constructed a new cell line derived from well-differentiated hepatocellular carcinoma tissues designated NHBL2. Immunol staining showed that NHBL2 possessed the capacity of synthesizing albumin and CYP2E1 and quantitative analysis showed that the albumin synthesis ability of NHBL2 was comparable to C3A while urea production was highly abundant of NHBL2 compared with that of C3A. Using gene expression microarray analysis, we found that the expression levels of a set of genes encoding Phase I and Phase II metabolizing enzymes as

well as many others related to common bioconversion and metabolic processes were significantly higher in NHBL2 cell line than that in C3A. Moreover, functional optimization assay in Matrigel showed obvious improvements of liver-related function level and a low malignance of this cell line. These findings indicated that NHBL2 possessed relatively attractive and full hepatic functionality that might be a potential cell line for BAL development.

Keywords Biomaterial · Cell isolation · Cell line · Liver function · Microarray · Three-dimensional culture

Introduction

Acute liver cell failure (ALF) is a life-threatening liver injury associated with a high mortality (Punzalan and Barry 2015) and medical treatment alone is insufficient to improve the outcome of these patients till now. Over the recent decades orthotopic liver transplantation (OLT) continues to give patients the best chance for cure (Fernandez et al. 2015), but severe shortage of available donor organs and increasing number of patients on waiting lists have promoted to searching for alternative effective ways (Bernhardt and Reichen-spurner 2014). Bioartificial liver systems (BAL) have been conceived to either bridge patients towards OLT or serve as potential supportive therapies for patients

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awaiting tissue regeneration (Starokozhko and Groothuis 2016).

Biomaterial played a crucial role in the bridge-function of BAL due to the complex metabolic and detoxification functions of cells in the bioreactor. Cryopreserved porcine cells and human primary hepatocytes have been the most frequently used as the biocomponent in BAL system (Demetriou et al. 2004; Lee et al. 2017). However, the risk of zoonoses of porcine cell (Sakiyama et al. 2017) and the shortage as well as the poor proliferation capacity of human primary hepatocytes (Liu et al. 2013) rendered it necessary to explore other sources of biomaterials. For these reasons, liver-tumor-derived cell lines have been extensively studied for development of the BAL. As a clonal derivative of HepG2, C3A cell line was the only hepatoma cell line that has been applied in clinical trials in patients with ALF. However, the ammonia detoxification capacity of C3A is poor rendering its limited application in BAL and there have been no further clinical research in the last few years (Mavri-Damelin et al. 2008). Hence, none of the present hepatoma cell lines are suitable for BAL application primarily due to their insufficient functionality (Yang et al. 2013) and it is necessary to search for the optimal alternative.

In this study, we carried out the construction of new cell lines derived from well-differentiated HCC tissues from a 36-year-old adult and successfully established a hepatoma cell line designated NHBL2. In order to evaluate its application in BAL, we detected their biological characteristics and liver function at transcript and biochemical level. Furthermore, microarray analysis was used to characterize the whole genome gene expression profiles of this new cell line. For functional optimization, NHBL2 was cultured in Matrigel for 7 days and the expressions of most hepatocyte-specific function genes showed different levels of improvement tested by q-PCR. This study provided an alternative cell source for BAL systems.

Methods

Cell lines and specimens

C3A was purchased from American type culture collection (ATCC, Zhongyuan, Beijing, China). The use of human liver samples for scientific research has

been approved by the Medical Ethics Committee of Nanfang Hospital, Southern Medical University on 15, March, 2012. All liver tumors were obtained from a total of 24 patients with an average age of 47 who underwent liver hepatectomies at the Department of Hepatobiliary Surgery, Nanfang Hospital. Relatively normal liver tissues located at distance from the lesion were collected as a control. All patients were pathologically diagnosed with hepatocellular carcinoma (HCC).

Cell isolation and culture

Primary hepatocytes were isolated by collagenase digestion as described by Selden et al. (2003). Briefly, the HCC fragment was perfused through the vascular orifices on the cut surfaces with Hanks Balanced Salt Solutions (HBSS, Gibco, Grand Island, NY, USA) at 37 °C for 15 min to remove the blood cells followed by further perfusion with collagenase type IV (Sigma, St. Louis, MO, USA) solution for another 15 min. Then the samples were minced into small pieces followed by the digestion with collagenase type IV solution at 37 °C to get intact cells released. The cell suspension was filtered through a sterile 100-μm nylon mesh, sedimented by centrifugation at 50 g for 5 min and washed twice with cold wash medium. The freshly isolated hepatocytes were seeded onto collagen-coated culture plates in DMEM supplemented with 10% fetal calf serum (FCS, Gibco), 100 IU/ml penicillin (Sigma), 100 μg/ml streptomycin (Sigma), 10^{-8} M insulin (Sigma, St. Louis, MO, USA), 10^{-8} M dexamethasone (Sigma) at 37 °C, 5% CO₂. Growing cell colonies were individually picked and amplified. Medium was changed twice a week and cell passages were performed with 0.25% trypsin (Gibco, Grand Island, NY, USA) when the cells got approximately 80% confluence. Finally, the immortalized cell line was established after 50 passages.

Cell density and growth curve analysis

Immortalized cells were plated into 6-well plates at an initial density of 2×10^4 cells/well in 2 ml DMEM containing 10% FCS. The cells were counted every 24 h for a total of 7 days and the cell growth curve was drawn with culture time as the abscissa and cell numbers as the ordinate. The cell doubling time (DT) was calculated using the formula $DT = t * [\log(2)/$

$(\log N_t - \log N_0)$], t = culture duration, N_t = final concentration, N_0 = initial concentration. C3A cell line was used as the control group.

Microarray analysis

The whole genome gene expression profiles of NHBL2 and C3A were determined by Microarray in triplicate replicates of each sample. Total RNA was extracted using the Trizol Reagent method. RNA samples were purified using RNeasy columns (Qiagen, Valencia, CA, USA, cat. no. 74104) and labeled according to the chip manufacturer's instructions. In brief, for Illumina, 0.5 μ g RNA from each sample was labeled using the Illumina Total Prep RNA Amplification Kit (Ambion, Austin, TX, USA, cat. no. IL1791) followed by RNA transcription. Single stranded RNA (cRNA) was generated and labeled by incorporating biotin-16-UTP. 0.75 μ g of biotin-labeled cRNA was hybridized (16 h) to Illumina Human HT-12_v4 BeadChips (Illumina, San Diego, CA, USA). The hybridized biotinylated cRNA was detected with streptavidin-Cy3 and quantified using Illumina's BeadStation 500GX genetic analysis systems scanner.

Preliminary data analysis of the scanned data was performed using Illumina BeadStudio software which returned single intensity data values/gene following the computation of a trimmed mean average for each probe type represented by a variable number of bead probes/gene on the array. Z-transformation for normalization was performed on each Illumina sample/array on a stand-alone basis, and significant changes in gene expression between class pairs were calculated by Z test. Significant gene lists were calculated by selecting genes which satisfied a significance threshold criteria of Z test P values less than or equal to 0.001 (10^{-3}), a false discovery rate less than or equal to 0.1, and a fold change ± 1.5 or greater.

Immunohistochemistry (IHC)

Immunohistochemical staining was conducted using standard procedure as previously described (Mhaidat et al. 2016). Briefly, the collected specimens were fixed with formalin (Tianyu, Guangzhou, China) followed by paraffin embedding and the staining was done on 4- μ m sections from the embedded tissue specimens. The sections were deparaffinized in

xylene, rehydrated by a series of graded ethanol rinse and heated in a microwave oven in 0.01 M sodium citrate buffer (pH 6.0) for 20 min to retrieve antigens. Endogenous peroxidase activity was terminated by incubation in 3% H_2O_2 for 20 min at room temperature. The sections were then incubated at 4 °C overnight with goat anti-human albumin (ALB) polyclonal antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat anti-human Cytokeratin 18 (CK18) polyclonal antibody (1:50; Santa Cruz Biotechnology), and rabbit anti-human Cytochrome CYP2E1 monoclonal antibody (1:50; Abcam, Cambridge, UK), respectively. Then the sections were incubated with the corresponding secondary antibodies (Zhongshan Biotechnology, Beijing, China). The antibody-antigen complexes were visualized with diaminobenzidine (DAB, Zhongshan Biotechnology) alone and counterstained with haematoxylin (Zhongshan Technology). Finally, the sections were dehydrated in ethanol, cleared in xylene and examined under a light microscopy.

Immunofluorescent (IF) and immunocytochemical (ICC) staining

Immortalized cells grown on coverslips were fixed with 3% formaldehyde for 20 min and blocked with 5% bovine serum albumin (BSA, Zhongshan Biotechnology) at room temperature for 1 h. Then cells were incubated at 4 °C overnight with the antibody mentioned above followed by incubation with Cy3-labeled goat anti-rabbit-IgG or rabbit anti-goat IgG secondary antibodies (EarthOx Life Science, Millbrae, CA, USA).

PAS staining

Cells grown on coverslips were fixed with 3% formaldehyde for 20 min, then the coverslips were stained using a PAS staining kit (Maixin. Bio, Fuzhou, China) following the manufacturer's instructions (Saraiva-Pava et al. 2015; Takahashi et al. 2014). The PAS-stained cells were counterstained with haematoxylin for 6 min, and finally examined under a light microscopy (IX73, Olympus, Beijing, China).

Measurement of albumin and urea productions

The immortalized hepatocytes were plated in 6 well plates at a density of 4×10^4 /well in 2 ml DMEM supplemented with 10% FCS and the supernatant was collected 2 days after cell attachment. To detect albumin production, the collected medium was analyzed by a human albumin ELISA Quantification Kit (R&D, Minneapolis, MN, USA) (Hu et al. 2016). To examine the urea synthesis, the collected medium was analyzed by a commercial BUN detection kit (Arbor Assays, MI, USA). C3A cell line was used as control.

Quantitative polymerase chain reaction analysis of gene expression

Total RNA was extracted from the immortalized hepatocytes and C3A cell line using TRIZOL[®] reagent (TaKaRa, Dalian, China) according to the manufacturer's instructions. Complementary DNA (cDNA) synthesis was performed by reverse transcription reagent kit (TaKaRa) according to the manufacturer's protocols. The reaction was held at 37 °C for 15 min and RTase was heat-inactivated at 85 °C for 5 s. Gene expression was thus measured on the Roche Light

Cycler 480 Real Time PCR System (Roche Diagnostics, Mannheim, Germany) using SYBR Green qPCR Master Mix (Takara). The reactions were carried out as follows: 40 cycles of 95 °C (30 s), 95 °C (5 s), and 60 °C (20 s) followed by one cycle of 65 °C (15 s). The primer sequences for real-time PCR are shown in Table 1. Relative gene expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method (Hu et al. 2016). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Invitrogen, Shanghai, China) was used as an internal reference.

Functional optimization assay in 3D Matrigel

A 24-well plate was coated with 20 μ l Matrigel (BD Bioscience, CA, USA) per well and the Matrigel matrix was polymerized at 37 °C for 30 min. The cells were collected and resuspended at a density of 8×10^4 /ml in DMEM containing 10% FCS. The cell suspension was mixed with Matrigel (1:1) and 1×10^3 cells were plated in each well of the pre-coated 24 well plate. For morphology observation, images of the immortalized hepatocytes in 3D Matrigel were taken every other day by an inverted optical microscope. For functional detection, RNA

Table 1 The upstream and downstream sequence of primers used for real time PCR

Primer	Primer sequences	
	Forward	Reverse
ALB	5-GCCTGCTGACTTGCCTTCATTAG-3	5-TCAGCAGCAGCACGACAGAGTA-3
TTR	5-CAGAAAGGCTGCTGATGACA-3	5-ATGCCAAGTGCCCTTCCAGTA-3
AAT	5-CAACCTGGCTGAGTTCGCCT-3	5-CTCGCTGAGGAACAGGCCAT-3
TF	5-CCCTTAACCAATACTTCGGCTAC-3	5-TTTGCCAAGTTCTCAAATATAGTCG-3
CYP2E1	5-CTACAAGGCGGTGAAGGAA-3	5-TCTCATTGCCCTGTTTCCC-3
G-6-P	5-GCTGCTCATTTTCCTCATCAA-3	5-TTCTGTAACAGCAATGCCTGA-3
GST- π	5-CCTGTACCAGTCCAATACCATCCT-3	5-TCCTGCTGGTCCTTCCCATA-3
CPS-1	5-TGAGGGATGCTGACCCCAT-3	5-CATTGTTGGCGTTGAGCCAG-3
TAT	5-AGGCCAGGTGGTCTGTGAGG-3	5-AGGGGTGCCTCAGGACAGTG-3
CYP3A5	5-CCTTACCCAGTTTTTGAAGCA-3	5-TCCAGATCAGACAGAGCTTTGTG-3
CYP3A4	5-CAGGAGGAAATTGATGCAGTTTT-3	5-GTCAAGATACTCCATCTGTAGCACAGT-3
GST- α	5-TGGCAGAGAAGCCCAAGCTC-3	5-TGCACCAGCTTCATCCCATC-3
AFP	5- GCTGACATTATTATCGGACAC -3	5-GAAGTGTGTCATCAGAGAATGC-3
CK-18	5-GAGACGTACAGTCCAGTCCTTGG-3	5- CCACCTCCCTCAGGCTGTT-3
HNF-1 α	5-TACACCACTCTGGCAGCCACACT-3	5-CGGTGGGTACATTGGTGACAGAAC-3
GAPDH	5-GAAGGTGAAGGTCTGGAGT-3	5-GAAGATGGTGATGGGATTTC-3

was extracted from the immortalized hepatocytes cultured in Matrigel for 7d, and q-PCR was performed as mentioned above. The immortalized hepatocytes cultured in regular cell culture dishes (2D) were used as a control group.

Statistical analysis

All statistical analyses were performed with SPSS 19.0. Statistical analyses of the data were performed using the Student's *t* test to establish the significance between data points and statistical significance was determined using a $P < 0.05$.

Results

Morphology and growth characteristics of NHBL2

Among the specimens of the patients included, we managed to extract the cell line from a 36-year-old male patient pathologically diagnosed with a well differentiated hepatocellular carcinoma. Initially, the isolated primary hepatocytes gained large and polygonal morphology in adherent culture, mixed with a few long-spindle interstitial cells. After several weeks, most primary cells underwent apoptosis while a continuous proliferation cell colony was observed which was picked for further cultivation (Fig. 1A). The separated cells gradually gained active proliferation capacity and an irregular polygonal cell

morphology smaller than primary hepatocytes after 50 passages which indicated the establishment of a hepatoma-derived cell line named new human-derived cell line for bioartificial liver (NHBL2). The cell growth curve was established by cell counting. As shown in Fig. 1B, NHBL2 began to enter the fast-growing period 72 h after adherence to the culture flasks with a cell doubling time of 27 h.

Microarray analysis

Based on the observations described above, we performed Illumina's whole-genome expression arrays to describe the gene expression profiling of NHBL2. The results showed that the expressions of most genes were at the same level between NHBL2 and C3A cells. As shown in Fig. 2, 177 out of 19,647 genes (0.9%) were upregulated and 71 out of 19,647 genes (0.36%) in NHBL2 cell line compared with that in C3A cells were downregulated by at least 3 times. Gene set enrichment analysis (GSEA) showed that these upregulated genes were mainly associated with cell cycle, proliferation, detoxification and metabolism-related signaling pathways. We further analyzed the genes annotated as being involved in detoxification and metabolism. As shown in Fig. 2, the expressions of genes encoding Phase I metabolizing enzymes (ALDH1A1 and ALDH9A1) as well as genes encoding Phase II metabolizing enzymes (GSTA1, GSTA2 and UGT1A1) were more than three to fourfold higher in NHBL2 compared with those in C3A cells.

Fig. 1 Morphology and growth characteristics detection: **A** Morphological observation of different cells during the development of NHBL2 cells: **a** the primary cells after isolation (200×), **b** cells 2 weeks after isolation (100×), **c** the

10th generation of NHBL2 (200×), **d** the established NHBL2 cell line (200×). **B** Growth curve of NHBL2 detected by cell counting

Fig. 2 Gene expression profiling of NHBL2: **A** the heatmap image of NHBL2 and C3A cell lines gene data. **B** The corrmatrix of NHBL2 and C3A cell lines gene data, the correlation of all samples was very high, over 0.98. **C** The

expression of most genes was at the same level between NHBL2 and C3A cells. **D** The results of microarray were further confirmed by q-PCR assay and GAPDH was used as an internal reference

Furthermore, the expression of a set of genes involved in common bioconversion and metabolic processes such as HMGCS1, SHMT1 and AMACR were also significantly higher (ranging from three to fivefold) in the NHBL2 cell line than that in the C3A cells. In addition, the expression level of most synthesis-related genes such as ALB, TTR, TF, was not significantly different between the two cell lines.

Functional examination of NHBL2 cell line

In addition to morphological observation, the liver function of NHBL2 was further analyzed. As shown in Fig. 3, Albumin, CK18 and CYP2E1 were extensively expressed in NHBL2. With regard to the localization, ALB and CK18 were predominantly detected in the cytoplasm and CK18 was mainly detected in intracytoplasmic cytoskeleton by ICC and IF analysis. The expression of AFP an important marker of hepatocellular carcinoma was more abundant in the C3A cell line than in the NHBL2. The expressions of genes

Fig. 3 Functional examination of NHBL2 cell line: **A** representative immunofluorescence images for Alb (1000 $\times$), CYP2E1 (400 $\times$) and CK18 (1000 $\times$) in the NHBL2 cell line. **B** Representative immunohistochemical images for ALB (a,

200 $\times$), CYP2E1 (b, 200 $\times$) and CK18 (c, 200 $\times$) in NHBL2 cell line. **C** The expression of liver specific genes was examined by q-PCR (* $P < 0.05$)

related to bioconversion and metabolism (CYP3A4, CYP3A5, GST- α , CPS-1) was higher in the NHBL2 cell line compared with that in C3A cells and expression of synthesis-related genes (ALB, TTR, AAT) was almost at the same level in accordance with the microarray analysis.

PAS staining showed homogeneous distribution of glycogen granules indicating the active glycogen synthesis and storage capacity of NHBL2 (Fig. 4A). To evaluate the ammonia detoxification and synthesis capacity, ALB and blood urea nitrogen (BUN) concentration in the supernatant of NHBL2 and C3A was analyzed in triplicate replicates. The results showed that the urea production in supernatant from NHBL2 cells was significantly higher compared with that from

C3A cells (3.78×10^{-6} vs 2.91×10^{-6} , $P = 0.047$) while albumin secretion analysis appeared not to be statistically different between the two cell lines (3.15×10^{-6} vs 2.5×10^{-6} , $P = 0.16$, Fig. 4B).

Functional optimization assay in 3D Matrigel

Matrigel could promote the differentiation of many different cell types. For functional optimization, cells were plated in Matrigel for 3D culture and the morphology was continuously observed. As shown in Fig. 5A, the cells gathered to form several spherical tight clumps in Matrigel. The cell colonies grew slowly in the first 2 days and gradually achieved active proliferation capacity from the third day onwards.

Fig. 4 **A** PAS staining of C3A (a, 400 $\times$) and NHBL2 (b, 400 $\times$). **B** Detection of ALB and BUN from culture supernatants of NHBL2 cell line (* $P < 0.05$)

Fig. 5 Functional optimization experiments in Matrigel: **A** NHBL2 cells cultured in three-dimensional Matrigel were continuously observed under an optical microscope. **B** The expressions of liver function-related genes of NHBL2 cultured

in three-dimensional Matrigel for a week were examined by q-PCR assay in comparison to the cells grown in regular cell culture dishes

After cultured in Matrigel for 7 days, the expressions of liver-related genes of the NHBL2 cell line was detected by q-PCR. As illustrated in Fig. 5B, the expression levels of more than half of the 12 liver-related genes (TTR, GST-pi, CPS-1, TAT, CK18, CYP3A5, GST- α) were significantly increased in NHBL2 cultured for in Matrigel compared with the cells cultured in regular cell culture dishes ($P < 0.05$) while the expressions of the other 5 genes (ALB, AAT, TF, CYP2E1, G6P) showed no statistical significance ($P > 0.05$).

Discussion

As we know, full hepatic functionality of the biomaterials in BAL is crucial for supporting patients with ALF. The ideal human hepatocyte cell line for BAL application should probably possess high proliferation capacity together with full hepatic functionality. For the high hepatic functionality, human primary hepatocytes are supposed to be the optimal source of ideal biomaterial. However, the short lifespan has hampered its use in BAL development (Guoliang et al. 2011). To overcome this issue, a liver-tumor-derived cell line was established in this study. As demonstrated in our previous study, the well differentiated HCC possessed more attractive liver function than the poorly differentiated one, some of which was to some extent

comparable with the relative normal liver tissues. Here, we selectively used the highly differentiated tumor tissues for cell isolation and finally successfully established a novel human cell line derived from well differentiated HCC tissues which could maintain a proliferative status in the standard culture medium *in vitro* with a cell doubling time of 27 h.

Given the high proliferation ability, many other tumor-derived human cell lines have been studied in BAL models. C3A cell line was a clonal derivative of HepG2 which was isolated from a 15 years male Caucasian adolescent. It has been used in the extra-corporeal liver assist device (ELAD) system to treat patients with ALF because C3A cells, in particular, are capable of producing urea and albumin (Ellis et al. 1996). Consistently, our previous study has shown that the hepatic functionality of the present human liver cell lines was generally absent or insufficient and C3A possessed the relatively better liver function among them. As a result, here we selected C3A as the control group to explore the function of NHBL2. We found that NHBL2 possessed the capacity of synthesizing albumin and CYP2E1 by IHC and ICC detection. Further quantitative analysis showed that the albumin synthesis ability of NHBL2 was comparable to C3A. Furthermore, NHBL2 positively expressed CK18 indicating the epithelial tissues origin of this cell line.

In BAL treatment, it is essential for the cells to metabolize various toxic substances including ammonia. Despite the active albumin synthesis capacity of C3A, their poor ammonia detoxification and mixed function oxidase activity has prevented it from demonstrating a survival benefit when randomized to standard medical therapy of liver failure. Our study showed that urea production was highly abundant in the supernatant of NHBL2 compared with that in supernatant of C3A.

As a powerful molecular technique, microarray analysis has been extensively used to characterize the gene expression profiling of different tissues or cell lines (Jennen et al. 2010). Using gene expression microarray analysis, we found that the expression levels of a set of genes encoding phase I and phase II metabolizing enzymes were significantly higher in NHBL2 cell line than that in C3A. These enzymes mainly function in the detoxification of carcinogens, therapeutic drugs, alcohol, hormones, bilirubin and products of oxidative stress. In addition to the phase I and II genes, many others related to common

bioconversion and metabolic processes such as lipid and cholesterol metabolism, fatty acid and amino acid conversion and beta-oxidation were identified prominently abundant in NHBL2 compared with C3A cells. However, the expressions of genes involved in synthesis-related functionality such as ALB, TTR, TF, etc. appeared no obvious difference between the two cell lines. Collectively, these findings indicated that NHBL2 cells possessed relatively attractive and full hepatic functionality thus the NHBL2 cell line might be a potential cell line for BAL development.

Matrigel is a soluble and sterile extract of basement membrane proteins that forms a 3Dgel at 37 °C and mimics the *in vivo* microenvironment of tumor cells and affects not only the morphology and tumor growth of the cells but also their differentiation process (Lam et al. 2017). Prior studies have shown that both primary and established cells reflected a more differentiated phenotype of the morphology and the genes expressed by the cells when exposed to this matrix. Furthermore, the morphology of the tumor cells in Matrigel is comparable to their malignant and invasive potential. Low metastatic and invasive cells will grow in tight clumps whereas malignant cells will grow to form a star-like appearance (Benton et al. 2009, 2014). In accordance with the published reports, in this study, expression of most of the liver-related genes of NHBL2 was improved in Matrigel compared with the cells cultured in regular cell culture dishes. Meanwhile the tight clumps appearance of NHBL2 cultured in Matrigel reflected a low metastatic and invasive potential of this cell line.

In conclusion, we constructed a new liver cell line NHBL2 derived from well-differentiated HCC tissue with a low metastatic and invasive potential. The unlimited lifespan and relatively full hepatic functionality rendered it a potential cell source for application to BAL although more comprehensive explorations of this cell line need to be performed.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflict of interest.

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