Title: Biomechanical profile of cancer stem-like cells derived from MHCC97H cell lines

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Abstract

Biomechanical properties and cytoskeletal organization of cancer cells are known to be closely related with their aggressive phenotype. In this study, based on atomic force microscopy (AFM), we aimed to evaluate the mechanical property of liver cancer stem-like cells (LCSCs) and compare it with human hepatoma cells (HHCs). LCSCs were enriched from human hepatoma cell line MHCC97H through a sphere culture system. AFM nanoindentation was investigated as a method for measuring the cell stiffness, and reflecting by the Young's modulus. Microfilament bundles of F-actin were observed with immunofluorescence staining by confocal microscopy. We found that LCSCs show lower Young's modulus and higher migration ability compared to MHCC97H cells. Moreover, the decrease in Young's modulus is accompanied with a dramatic decline in F-actin content. These results demonstrated a close relationship between the cell Young's modulus and metastatic potential of HHCs, which suggest that the Young's modulus detected by AFM can be used to evaluate metastatic potential of cancer cells.

Keywords: Liver cancer stem-like cells; Human hepatoma cells; Atomic force microscopy; Young's modulus; Metastatic potential

Abbreviations: HHCs, human hepatoma cells; LCSCs, liver cancer stem-like cells; AFM, Atomic force microscopy; SFCs, sphere-forming cells; TSM, tumor sphere medium

1 Introduction

Cancer is the second leading cause of death worldwide. Metastases and drug resistance phenotypes almost result in poor prognosis of conventional cancer treatment and high possibility of recurrence. It is hypothesized that a rare subset of cancer cells, often operationally referred to as “cancer stem cells (CSCs)”, are responsible for sustaining cancer metastasis and recurrence (Rosen and Jordan, 2009). Therefore, they are generally more resistant to chemotherapeutics compared to their fully differentiated tumor cell counterparts (Lapidot et al., 1994; Park et al., 1971). The current challenge is to determine the self-renewal capacity of individual tumor cells in order to assess the validity of either model in a specific tumor cell type.

The occurrence and progression of cancer can be strongly linked to mechanical properties of individual living cells. It has been revealed that deviations in the mechanical properties of living cells may play important roles in many cell physiological events, such as cell differentiation (Fishkind and Wang, 1995), cell deformability (Dartsch et al., 1994), invasion and metastasis of cancer cell (Suresh, 2007a). Other studies claimed that cancer can induce mechanical property changes in cells, which may be a potential biomarker in the early detection of cancer and for anti-cancer drug efficiency test (Lee and Lim, 2007). Therefore, mechanical properties of individual cell may play a vital role in the study of cancer and tell high-malignant cancer cells from low-malignant ones or non-malignant ones (Crick and Yin, 2007). Furthermore, understandings of the mechanical properties of cancer cells are necessary to better understand the physical mechanisms responsible for cancer metastasis. It provides a potential clinical method for detection, diagnosis, and treatment of cancer. To date, mechanical properties of cells have been studied by different single-cell techniques, including magnetic twisting cytometry (Planus et al., 2002), atomic force microscopy (AFM) (Binnig et al., 1986), micropipette aspiration (Hochmuth, 2000), microfluidic techniques (Song et al., 2009), optical tweezers (Dao et al., 2003), scanning acoustic microscopy (Bereiter-Hahn et al., 1995). At present, AFM is one of the most advanced techniques used to study elastic properties of cells (Radmacher et al., 1996; Palmieri et al., 2015). AFM as a technique has many strengths, including that it is a small and easy to use instrument. AFM modes can work perfectly well in fluids and physiological conditions, it is relatively nondestructive to samples (e.g.

no fixing, dehydrating, staining, freezing or coating), it generates images in three-dimensions with high resolution, and it can be used to mechanically probe samples. AFM can also be combined with a variety of optical microscopy techniques such as fluorescent microscopy, furtherly expanding its applicability. As with any technique, there are some weaknesses when using AFM. Piezo actuators have a limited range in the z-direction, meaning that sample features typically must be less than 20 μm deep. In addition, since the probe is “feeling” the surface, there is the possibility that the shape of the probe is convoluted into the resulting image. This problem is made worse as a tip is dulled or damaged with imaging. Finally, another limitation of AFM-based cell measurement is that typical experiments are highly time and user-intensive, so that a limited number of cells can be analyzed in one day. Despite these limitations, there is much that this technique can do to push the cancer research field forward. AFM has also been used to investigate the mechanical properties of ex vivo cancer cells obtained from patients (Cross et al., 2007). Previous studies have shown that the stiffness of embryonic stem cells is considerably softer before undergoing cellular differentiation process (Chowdhury et al., 2010; Pajerowski et al., 2007; Titushkin and Cho, 2007). Also, a recent study using a mechanical separation microfluidic chip has shown that breast tumor-initiating cells (TICs) exhibit a more uniform deformability compared to more differentiated cancer cells (Zhang et al., 2012). However, it has yet to be clarified potential differences or similarities between the mechanical properties of CSCs and its normal cancer cell counterparts. Here, we use human hepatoma cell line MHCC97H and CSCs derived from MHCC97H to build a possible evaluation method to explain the relationship between metastatic potential of LCSCs and the Young’s modulus by AFM.

2 Materials and methods

2.1 Cell culture

The human hepatoma cell line, MHCC97H was obtained from the Liver Cancer Institute, Zhongshan Hospital, Fudan University (Shanghai, China), were maintained as monolayer cultures in high glucose DMEM supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA), penicillin (100 U/mL), and streptomycin (100 $\mu\text{g/mL}$), and in 37°C, 5% CO₂ incubator.

2.2 Enrichment of sphere-forming cells (SFCs)

LCSCs appear to be spherical, so we termed it sphere-forming cells (SFCs). SFCs were derived by placing the MHCC97H cells grown as monolayers into tumor sphere medium (TSM) consisting of DMEM/F-12 medium supplemented with B27 supplement without vitamin A, 1% N2 supplement, 20 ng/mL epidermal growth factor, 10 ng/mL basic fibroblast growth factor (Life Technologies, Carlsbad, CA, USA). The culture media were replaced every 3 days. When primary tumor spheres formed after 5-8 days, cells were collected by gentle centrifugation, then dissociated enzymatically (3 min in 0.1% trypsin and 1 mM EDTA) and plated the cells from the tumor spheres in 6-well plates at a clonal density of 1000 cells/mL in TSM to acquire the secondary tumor spheres.

2.3 Characterization of SFCs derived from MHCC97H cells

2.3.1 Sphere formation assay

The third-generation spheres were dissociated as describe above, the dissociated SFCs were also diluted to 500 cells/mL. Then, the 2 μ L/well diluted cell suspension was plated to ultra-low attachment 96-well plate, and 100 μ L of serum-free medium was added. Cultures were fed 0.02 mL of TSM every 2 days until day 13. The wells with only one cell were marked and observed every day.

2.3.2 Chemotherapy sensitivity assay

The sensitivity of the MHCC97H cells and SFCs to chemotherapeutic drugs was measured by MTT assay. Briefly, cells were seeded at a density 3×10^3 cells /well in a 96-well culture plate that were precoated with Matrigel (Becton Dickinson Labware, Bedford, MA), and various concentrations of cisplatin, 5-FU(Sigma-Aldrich, Saint Louis, MO, USA) for 24 h. Cisplatin or 5-FU was added to the cell medium at a final concentration of 3 μ g/mL, 6 μ g/mL, 9 μ g/mL and 100 μ M, 200 μ M, 400 μ M, respectively. After 24 h of treatment, cell viability was evaluated by MTT (3-(4, 5-Dimethylthiazol-2yl)-2, 5-diphenyltetrazoliumbromide) assay (Sigma-Aldrich, Saint Louis, MO, USA).

2.3.3 Lactate dehydrogenase (LDH) assay

Cell death was evaluated by the quantification of plasma membrane damage which resulted in the release of lactate dehydrogenase (LDH). In brief, MHCC97H cells and LCSCs were cultured in 96-well plates. After cisplatin or 5-FU treatment for 24 h in DMEM supplemented with 0.25% BSA, the

level of LDH released in the cell culture supernatant was detected by LDH cytotoxicity assay detection kit (Beyotime, Jiangsu, China) following the manufacturer's instructions.

2.3.4 Colony formation assay

For analysis of colony formation, MHCC97H cells and SFCs were plated in 6-well plates that pre-coated Matrigel at a density of 2000 cells/well. After incubation for 2 weeks at 37°C, the colony formation ability was assessed by counting the number of colonies (> 70 cells) under a microscope after crystal violet staining. Representative views were photographed. MHCC97H cells were plated at the same density as the control.

2.3.5 Flow cytometry

The following anti-human monoclonal antibodies (mAbs) were used for flow cytometry: anti-Oct3/4-PerCP/Cy5.5 (Becton Dickinson Labware, Bedford, MA); anti-CD133-APC (Miltenyi Biotec, Bergisch Gladbach, Germany). Appropriate isotype controls were used for each antibody. Intracellular Oct3/4 staining was done after fixation and permeabilization with Fix/Perm buffers according to the manufacturer's instructions. Flow cytometry was performed using a BD AccuriTMC6 flow cytometer (Becton, Dickinson and Company, USA) equipped with software. Side scatter and forward scatter profiles were used to eliminate cell doublets. Results are expressed as fluorescence intensity (MFI) and as the % of positive cells.

2.4 Atomic force microscopy (AFM) analysis

The MHCC97H cells and SFCs were seeded at a density 3×10^3 cells in a 23 mm glass slide that were precoated with Matrigel. The cell stiffness measurements were carried out by an atomic force microscope (JPK, Berlin, Germany) in the contact mode and a soft V-shaped silicon nitride cantilever with a nominal spring constant of 0.03 N/m was used. A pyramid probe (TR400PSA; Olympus, Japan) with a side-angle of 17.5° was attached to the cantilever tip and measurements were recorded at the center of the cell with a tip scanning frequency of 1.0 Hz. During testing, the probe slowly approached the cell surface, contacted it, retracted, and the force–distance curves were obtained by recording the cantilever deflection and displacement of the probe driver during the approach-retract cycles. The force–distance curve is linear before the probe contacts the cell, and

then the curve starts to climb from zero with increasing curvature. The non-linear force-distance curve reflects the mechanical properties of the cell.

the Young's modulus for the cells was computed using the Hertz model (Lim et al., 2006). According to this model, the relationship between the applied force, F , and the indentation depth, δ , can be expressed as:

$$F = \frac{E}{1 - \nu^2} \frac{2 \tan \alpha}{\pi} \delta^2$$

Where E is the elastic or Young's modulus, ν is the Poisson ratio (assumed to be 0.5), and α is the opening angle of the probe. Young's modulus can be calculated from this expression.

In our experiments, thirty cells were randomly selected from each group, and individual cells were chose five points and measured five times each point. All measurements were performed at 37°C, and the cells were completely immersed in PBS buffer. The local elasticity of the cells was quantitatively determined from the force versus distance curves.

2.5 Immunostaining

The MHCC97H cells and SFCs were prepared using a protocol similar to that described above for AFM analysis. Then, cells were fixed with 4% formaldehyde in 1×PBS for 60 min and permeabilized with 0.25% TritonX-100 in 1×PBS for 10 min. After saturation with 5% bovine serum, the cells were incubated with TRITC-phalloidin (Sigma-Aldrich, Saint Louis, MO, USA) at 4°C overnight. The nuclei were labeled with DAPI for 10 min at room temperature. The cells were photographed with a confocal microscope (Leica, Solms, Germany). Fluorescence intensity of F-actin in each cell was analyzed using software (Image Pro-Plus, Mediacy, Inc., Rockville, MD, USA).

2.6 Electrophoresis and Western blot analysis

MHCC97H cells and SFCs were with 1×PBS and digested immediately. The proteins were extracted using a cell lysis buffer. After electrophoretic separation by 10% SDS-polyacrylamide gel electrophoresis, the proteins were electrotransferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were then blocked with Tris-buffered saline containing 0.1% Tween-20 (TBST) and 5% skim milk for 1h at room temperature. An F-actin

rabbit Ab (Abcam, USA) and GAPDH rabbit mAb (Cell Signaling Technology, Danvers, MA, USA) were used according to the manufacturer's protocols, and the membranes were incubated overnight with these antibodies at 4°C with slight shaking. Thereafter, the membranes were washed three times in TBST, and the signals were developed using a super chemiluminescence kit (ThermoFisher). A semiquantitative evaluation of the bands was performed by densitometry (VersaDoc, Bio-Rad, Hercules, CA, USA). The levels of F-actin were determined through their normalization to the protein level of GAPDH.

2.7 Transwell migration assay

The migration of MHCC97H cells and SFCs were performed using the transwell insert (Millipore, Billerica, MA) with polycarbonate membranes of 8 µm pore size. Briefly, 200 µL serum-free medium containing 2×10^4 cells were seeded into the inner part of insert. The inserts were placed into 24-well culture plate containing 600 µL medium with 1% FBS as chemoattractant. After 6 h incubation at 37°C in 5% CO₂, non-migration cells were scraped from the upper surface of the membrane with a cotton swab, and migrated cells remaining on the bottom surface were stained with 0.05% crystal violet in PBS for 30 min. Cells on the underside of insert were counted under a light microscope in 4 randomly-selected fields for each insert. The number of the cells in each field was counted and averaged.

2.8 Statistical Analysis

All data were expressed as mean $\pm$ standard deviation. The data of each group were analyzed by one-way analysis of variance (ANOVA) followed by t-test for significant differences between two groups, and a value of $p < 0.05$ was considered to be statistically significant.

3 Results

3.1 The parental MHCC97H cells could form self-renewing spheres, and possess stemness properties

To determine if a population of self-renewing cancer stem-like cells exist within the cultured HCCs, an enrichment method of CSCs should be established. Although there are currently different methods for the separation or enrichment of CSCs, we consider defined serum-free culture

condition is a powerful approach. The strength of this method is the ability to define the correct environment require to maintain the stemness of the CSCs. This method has been successfully used to select and expand cell populations with CSC characteristics from colon, lung cancers and gliomas. Moreover, EGF that we add to TSM was essential to support proliferation of CSCs, such as colon cancer stem cells (Feng et al., 2012), neuroblastoma initiating cell (Hansford et al., 2007) and hepatic stem-like cells (Li, et al., 2015). In our study, MHCC97H cells were cultured in the presence of 10% FBS as monolayers of adherent cells (Figure 1AI) and subsequently in TSM. After 13 days, nonadherent, three-dimensional tumor spheres appeared (Figure 1AII). In order to confirm the finding that a sphere could be generated from a single cell, SFCs were plated to a 96-well plate and the wells with one cell were visualized every day. Figure 1B showed the process of single SFC forming a sphere, tumor subspheres were evident after 13 days, displaying the self-renewing and proliferative capacity.

Cancer stem-like cells have been shown to be more resistant than non-stem cancer cells to chemotherapy. To examine whether a difference in the chemosensitivity of MHCC97H and SFCsexists, we performed a MTT assay by exposing 3,000 cells from each group in 96-well plates and treated with various concentrations of cisplatin and 5-FU. After 24 h, The viability of SFCs were higher under the treatment of 3 µg/mL, 6 µg/mL and 9 µg/mL cisplatin, being 1.5-fold, 1.5-fold, 1.4-fold, respectively, compared with the parental MHCC97H cells ($P < 0.05$) (Fig. 1C). In the case of 5-FU treatment (100µM, 200 µM, 400 µM), the viability of SFCs was again significantly higher than that of their adherent counterparts (1.4-fold, 1.7-fold, 2-fold, respectively) (Fig. 1C). The results support a role for these SFCs in chemoresistance, which may explain why current therapies fail to eradicate progenitors and prevent tumor regrowth. Additionally, LDH assay further to demonstrate 5-FU and Cisplatin have the more cytotoxicity to MHCC97H cells.

To further confirm that SFCs have stem cell-like properties, we performed colony formation assays to determine their in vitro self-renewal ability. The third-generation spheres and MHCC97H efficiently formed colonies in 2 weeks after growing in 6-well plates that pre-coated Matrigel (Fig. 1D). Both of them were adherently plated and were alive, but were unable to form comparable colonies. The SFCs proliferated significantly faster and induced more tumor colonies than

MHCC97H cells did. Colonies of 2,000 seeded MHCC97H/SFCs were $161.67 \pm 16.13 / 278.33 \pm 18.70$ ($P < 0.01$) (Fig.1D). The result strongly indicate that SFCs possess the capability of maintaining their high clonogenicity. Oct3/4 is a member the POU family of transcription factor and is known to play a pivotal role in the maintenance of self-renewal and pluripotency in ESCs (Nichols et al., 1998). Of interest, accumulating evidence indicates that the expression of Oct3/4 transcription factors have a strong correlation with cancer stem-like cells, such as liver (Tomuleasa et al., 2009), breast (Kim et al., 2011) and lung (Hu et al., 2008) cancer cells. Therefore, LCSCs related proteins, including CD133, Oct3/4 were examined by flow cytometry. The results found that CD133 and Oct3/4 were significantly enriched in SFCs compared with the MHCC97H cells (Fig.1E), indicating that SFCs possess LCSCs properties.

3.2 Young's Modulus of SFCs were less than MHCC97H cells

Cell stiffness, which is characterized by Young's modulus. AFM force-distance curves for HCCs were shown in Fig. 2A, 2B, the red curves represent extending curves, the blue curves represent retracting curves and the green curves were fitting curves of the extending curves. The average values of Young's modulus were showed in Fig. 2C, MHCC97H (0.7305 ± 0.196 Kpa) and SFCs (0.5824 ± 0.0996 Kpa). The approaching curve was converted into a fitting extending curve originating from the point of contact. An indentation curve ranging from 0 nm to 400 nm was used to compute the Young's modulus. These results suggest that MHCC97H cells were stiffer than the SFCs ($P < 0.01$).

3.3 Actin cytoskeletal of SFCs differs to MHCC97H cells

The dynamic regulation of the filamentous actin cytoskeleton (F-actin) is crucial to cell stiffness. In this study, we sought to determine whether varying of cell stiffness is owes to changing in actin cytoskeletal. Here, we compared the formation of F-actin in SFCs and MHCC97H by staining F-actin with TRITC-conjugated phalloidin. Additionally, western blotting was used to further compare protein levels between MHCC97H cells and SFC cells. We found that SFCs had less well-defined stress fibers than MHCC97H and hence exhibited a weaker actin filamentous network ($P < 0.01$) (Fig.3A, 3B). Moreover, WB result demonstrated that SFCs had less F-actin content than MHCC9H cells and hence exhibited a weaker actin filamentous network (Fig. 3C) ($p < 0.01$). These results

suggested that lower Young's Modulus of SFCs was related to the lower organization and decreased intensity of F-actin.

3.4 Migration of SFCs were stronger than MHCC97H cells

Change in cell stiffness, which is characterized by Young's modulus, has been found to be coupled with change in cell migratory behavior. To investigate the relation between cell migration ability and Young's Modulus, transwell migration assay was performed. Fig. 4A shows the representative images of crystal violet labeled SFCs and MHCC97H migrated to the bottom of transwell insert, respectively. Fig. 4B shows the quantitative cell number counted by invert microscopy at the bottom of transwell, corresponding to the images showed in Fig. 4A. These results indicated that cells with higher metastatic potential had lower Young's modulus values than cells with lower metastatic potential, thereby suggesting that cells with higher metastatic potential were softer.

4 Discussion

AFM measurements of cancer cell stiffness, reflected by the Young's modulus, have shown a strong correlation between cell deformability and cell malignancy (Guck et al., 2005). Studies that are in vivo and in vitro by AFM showed that some cancerous cells are softer than their normal counterparts. Lekka et al. used the atomic force microscope (AFM) to study the elasticity of normal human bladder epithelial cells (Hu609 and HCV29) and cancerous ones (Hu456, T24, and BC3726) by performing AFM indentation on these cells [6]. Normal cells are found to have an order of magnitude stiffer than cancer cells and this was attributed to the reorganization of the cytoskeleton. Similarly, Li et al. found that cancerous MCF-7 cells are less stiff and easier to deform than non-malignant MCF-10A cells due to the changes to the subcellular structures. Additionally, lots of researches indicate that malignant tumor cells are softer as compared to low-malignant or non-malignant tumor cells (Fuhrmann et al., 2011; Guck et al., 2005; Ketene et al., 2012; Suresh, 2007b). In recent years, researchers have also shown that mechanical properties are a crucial determinant for the efficiency of cancer cell metastatic (Fuhrmann et al., 2011; Guck et al., 2005; Ketene et al., 2012; Nikkhah et al., 2010; Swaminathan et al., 2011). Swaminathan et al. reported that cancer cells with the highest migratory and invasive potential are five times less stiffer than cells with the lowest

migratory and invasive potential (Swaminathan et al., 2011). Another research shows that chondrosarcoma mechanical properties may differ significantly among cell lines that exhibit different levels of aggressiveness and metastatic potential, with the most aggressive and invasive cell line, JJ012, showing the lowest modulus (Darling et al., 2007). Similar to above studies, we also found that LCSCs enriched from MHCC97H cells exhibit a higher metastatic potential and significant lower Young's modulus values than MHCC97H with lower metastatic potential. A strong relevance between the Young's modulus and metastatic potential was also found. Thus, these results indicate that the cellular Young's modulus may be relevant to the metastasis of HCCs and can be used as a cell marker and a diagnostic parameter for liver cancer cell metastasis.

The cytoskeleton, an internal polymer framework, is known to determine morphology and mechanical rigidity of cell. The cellular tensegrity theory indicates that tensional forces are borne by cytoskeletal microfilaments and intermediate filaments (Ingber, 2003). F-actin, which provides the highest resistance to deformation among cytoskeletal microfilaments and reflects the overall cell mechanical properties, is less expressed in metastatic cancer cells than in their nonmetastatic counterparts (Guck et al., 2005; Rolli et al., 2010; Suresh, 2007a). The reorganization of F-actin results in overall changes in mechanical properties of the cell, Guck et al. found that the increased deformability (i.e., a decrease in the Young's modulus) of cancer cells is accompanied with reduction in the amount of F-actin (Guck et al., 2005). Compared to tongue squamous cell carcinoma (TSCC) with lower metastatic ability, Zhou et al. indicated that decreased Young's modulus of TSCC cells with high metastatic ability was accompanied with decrease of cytoskeleton (F-actin), epithelial mesenchymal transition (EMT) (Zhou et al., 2013). In our study, we found that differences in cell cytoskeleton (F-actin) were accompanied with changes in the cell migration ability and Young's modulus. LCSCs with lower Young's modulus and higher migration ability had a reduced and less organized cytoskeleton. These results confirm that cytoskeleton changes may regulate the cellular Young's modulus and induce cell migration.

Many studies also suggest that CSCs directly contribute to the initiation of metastases has gained increasing acceptance. Al-Hajj et al. demonstrated that express high levels of CD44 breast cancer cells have enhanced metastatic capabilities (Al-Hajj et al., 2003). Hermann et al. showed that a

CXCR4⁺ subpopulation within CD133⁺ CSCs isolated from a metastatic pancreatic cell line, and that in human pancreatic cancer the presence CXCR4⁺ cells was associated with higher frequency of lymph node metastasis (Hermann et al., 2007). These results can be interpreted to suggest that a subset of CSCs has a higher metastasis ability than their normal cancer cells. On the other hand, cancer can result in structural and mechanical property changes in individual living cells. Babahosseini et al. study shows that ovarian surface epithelial (MOSE) cancer CSC/TICs populations display significantly less stiffness compared to that of the corresponding parental MOSE cells. Also, a recent study using a mechanical separation microfluidic chip has shown that breast TICs exhibit amore uniform deformability compared to more differentiated cancer cells (Zhang et al., 2012). Altogether, these results demonstrate that the elastic modulus profile of CSCs is unique and responsive to anti-cancer treatment strategies that impact the cytoskeleton architecture of cells. Above all, our results reveal a close relationship between the cellular mechanical properties and the metastatic potential of liver cancer cells. AFM nanoidentation is time saving for researching migration assays, determining the Young's modulus and enableing broad screening of suspicious lesions of LCSCs with metastasis. Moreover, this study also validates the mechanical properties of CSCs derived from breast cancer lines(Zhang et al., 2012), murine ovarian cancer cell lines (Babahosseini et al., 2014). Thus, our research increase the chance for obtaining distinctive cell biomechanical profiles, intending to provide means for effective cancer detection and treatment control. We believe that Young's modulus detected by AFM can potentially be applied to assess the aggressive phenotype of LCSCs and provide a novel diagnostic parameter for LCSCs metastasis.

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360 **Conflict of interest statement**

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362 The authors declare no financial or commercial conflict of interest.

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Figure Legends

Figure 1. Enrichment and characterization of LCSCs from MHCC97H. (A) MHCC97H form 3-D spheres in TSM, I: MHCC97H; II: SFCs. Scalebar=200 μ m. (B) Generation of a sphere from a single MHCC97H cell. The propagation of a single cell was recorded at day 1, 3, 5, 7, 9 and 13, separately. Scalebar = 100 μ m. (C) The SFCs showed a drug resistance phenotype. The MHCC97H and SFCs were treated with cisplatin and 5-Fu for 24 h. Cell survival was determined by MTT assay (n=3, * P < 0.05; ** P < 0.01). (D) The LDH released in the supernatant of MHCC97H and SFCs were treated with cisplatin and 5-Fu for 24 h (n=3, * P < 0.05). (E) Comparison of clonogenicity of the MHCC97H and SFCs in vitro. SFCs with higher clonogenicity than MHCC97H (means $\pm$ SD; n=3; ** P < 0.01). (F) SFCs were enriched for cells expressing CD133, Oct3/4 higher than the MHCC97H cells. (means $\pm$ SD; n=3; ** P < 0.01, *** P < 0.001).

Figure 2. Atom force microscopy (AFM) analysis revealed the differences in cell stiffness as evaluated by Young's modulus. (A) AFM force-distance curves in MHCC97H cells; (B) AFM force-distance curves in SFCs; (D) MHCC97H with higher expression of F-actin when compared with SFCs. Red curves are for extending curves, representing elasticity, blue curve are for retracting curves, representing viscosity and green curves are fitting curves of extending curves. The data are expressed as the means $\pm$ SD; n=30; ** P < 0.01.

Figure 3. SFCs have less F-actin content than the MHCC97H. (A) Immunofluorescence staining of F-actin (Red) and nucleus (blue) of MHCC97H cells (left) and SFCs (right). (B) The representative bands show the average optical density of F-actin. (C) SFCs have less F-actin content than the MHCC97H. Data are expressed as the means $\pm$ SD; n = 3; ** p < 0.01 compared with the MHCC97H.

498 **Figure 4.** SFCs have stronger migration ability than the MHCC97H. (A) Images of the migration of
499 MHCC97H cells and SFCs. Scalebar = 100 μ m.(B) Quantitation of MHCC97H cells and SFCs
500 migration . The data are expressed as the means $\pm$ SD; n=3; ***P<0.001.

Figure 1.

Figure 2.

Figure 3.

Figure 4.