



<b>Title</b>	<b>Fusion with stem cell makes the hepatocellular carcinoma cells similar to liver tumor-initiating cells</b>
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<b>Citation</b>	<b>BMC Cancer, 2016, v. 16 n. 1, article no. 56</b>
<b>Issued Date</b>	<b>2016</b>
<b>URL</b>	<b><a href="http://hdl.handle.net/10722/223858">http://hdl.handle.net/10722/223858</a></b>
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RESEARCH ARTICLE

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# Fusion with stem cell makes the hepatocellular carcinoma cells similar to liver tumor-initiating cells

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## Abstract

**Background:** Cell fusion is a fast and highly efficient technique for cells to acquire new properties. The fusion of somatic cells with stem cells can reprogram somatic cells to a pluripotent state. Our research on the fusion of stem cells and cancer cells demonstrates that the fused cells can exhibit stemness and cancer cell-like characteristics. Thus, tumor-initiating cell-like cells are generated.

**Methods:** We employed laser-induced single-cell fusion technique to fuse the hepatocellular carcinoma cells and human embryonic stem cells (hESC). Real-time RT-PCR, flow cytometry and in vivo tumorigenicity assay were adopted to identify the gene expression difference.

**Results:** We successfully produced a fused cell line that coalesces the gene expression information of hepatocellular carcinoma cells and stem cells. Experimental results showed that the fused cells expressed cancer and stemness markers as well as exhibited increased resistance to drug treatment and enhanced tumorigenesis.

**Conclusions:** Fusion with stem cells transforms liver cancer cells into tumor initiating-like cells. Results indicate that fusion between cancer cell and stem cell may generate tumor initiating-like cells.

**Keywords:** Cell fusion, Hepatocellular carcinoma, Stem cell, Tumor-initiating cell

## Background

Cell fusion is a cellular process of combining two or multiple cells to form a single entity. Today, fusion with stem cells is used to induce somatic cell nuclei reprogramming and study the cell reprogramming mechanism [1]. Cell fusion is also a fast and efficient approach to induce pluripotent stem cells. The ability of somatic cells to acquire homologous characteristics and be converted to pluripotent cells with minimal modification have been demonstrated through the fusion of stem cells [2–4].

Hepatocellular carcinoma (HCC) is a malignant tumor with high mortality rate. New therapeutic strategies

against liver cancer are widely discussed in research and clinical medicine. Stem cell research is a potential approach to liver cancer treatment [5]. Examples of these approaches include the injection of stem cell for damage repair [6], liver regeneration [7], and induced differentiation of hepatocyte cells [8].

This paper presents a study on engineered fusion of stem cells and cancer cells to explore mechanism of cancer stem cell that may produce potential influence to stem cell therapy for liver cancer treatment. Through laser-induced fusion between a single hepatocellular carcinoma cell (HepG2) and a human embryonic stem cell (hESC), we determined that the fused cell acquired stemness and cancer-like characteristics. The fused cells exhibited increased tumorigenicity and resistance to drug treatment. The gene expression analysis indicated that the fused cells were highly similar to tumor stem cells (TSCs) or tumor-initiating cells (TICs). TICs are important in tumorigenesis, aggravation, metastasis,

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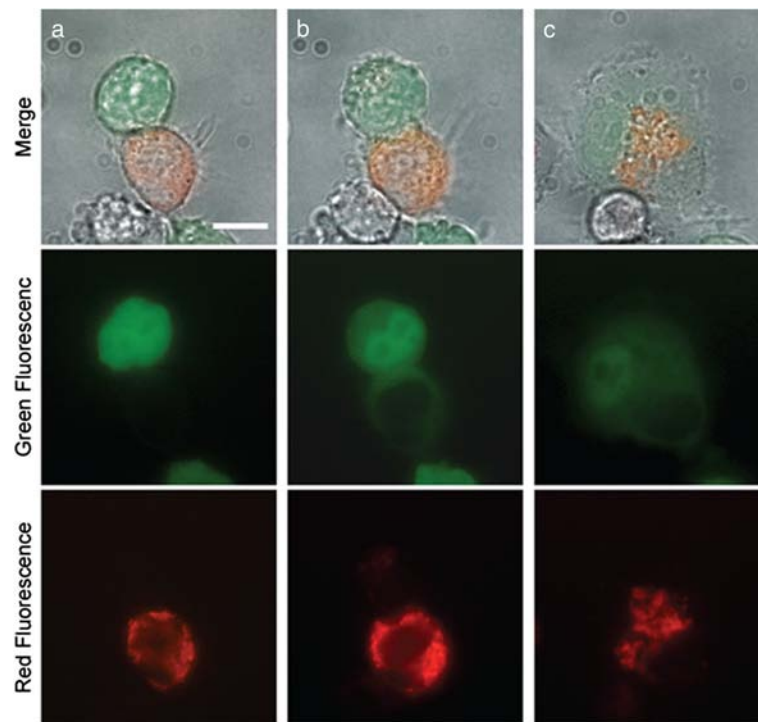
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**Fig. 1** Laser-induced cell fusion of hESC and HepG2. **a** GFP-labeled hESC (green) and mitochondria-labeled HepG2 (red) forming a cell pair before laser cutting. **b** GFP transfer from the green cell to the red cell after laser cutting. **c** The fused cell attracted to the coverglass surface and exhibited adherent cell morphology about 25 min after laser cutting. (Scale bar = 20 $\mu$ m)

and recurrence [9, 10]; liver TICs and their markers were identified [11–13]. Based on the results of gene expression analysis and quantity PCR detection, we found that some liver TIC markers such as CD133, CD44, and ALDH1 exhibited significantly high expression in the fused cells. However, this condition was not observed in HepG2 cells. The *in vivo* tumorigenicity assay and drug resistance assay also demonstrated that the fusion of cancer cells with stem cells resulted in cancer cells that were highly similar to cancer stem cells. These results suggested the possibility of incurring risks when some stem cell therapies are used for cancer treatment.

## Methods

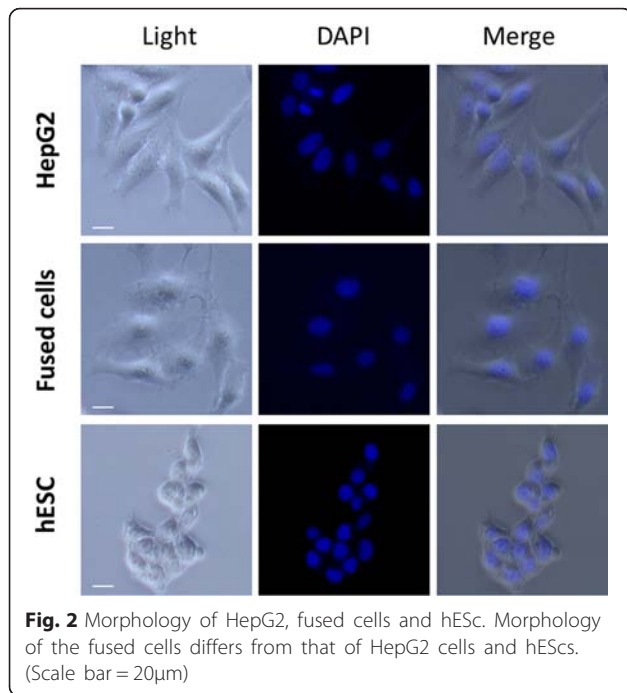
### Cell culture

The hESC line HES2 (NIH code: ES02) from ES Cell International (passage 60 to 84) was cultured on a commercially available, serum-free, and feeder-independent system (mTeSR1, Stemcell Technologies) and supplemented with BD Matrigel hESC-qualified Matrix (BD Catalog #354277), as suggested by the manufacturer. Genetically labeled *Oct4*-GFP<sup>+</sup> hESCs were generated by transduction (MOI = 5) with lentiviral vectors carrying a cassette of *Oct4* promoter-driven GFP.

HepG2 cells were maintained in DMEM (Gibco, Catalog #11965-092), supplemented with 10 % fetal bovine

**Table 1** Specific primers used in qPCR

Gene	Forward primer (5'-3')	Reverse Primer (5'-3')
AFP	AGACTGAAAACCTCTTGAATGC	GTCCTCACTGAGTTGGCAACA
CD133	ACATGAAAAG ACCTGGGGG	GATCTGGTGTCCCAGCATG
CD44	TCCCAGACGAAGACAGTCCCTGGAT	CACTGGGGTGGAAATGTGTCTTGGTC
ALDH1A1	CTGCTGGCGACAATGGAGT	CTGCTGGCGACAATGGAGT
ABC B1	GGGAGCTTAACACCCGACTTA	GCCAAAATCACAAGGGTTAGCTT
EpCAM	AATCGTCAATGCCAGTGTACTT	TCTCATCGCAGTCAGGATCATAA
Bcl-2	GACTGAATCGGAGATGGAGACC	GCAGTTCAACTCGTCGCCT
$\beta$ -actin	CATCCTCACCTGAAGTACCC	AGCCTGGATAGCAACGTACATG



serum (FBS, Gibco, Catalog #10270-106), 100 U/mL penicillin, and 100 U/mL streptomycin (Invitrogen, Catalog #15240-062) at 37 °C in a humidified atmosphere of 5 % CO<sup>2</sup>.

**Cell fusion**

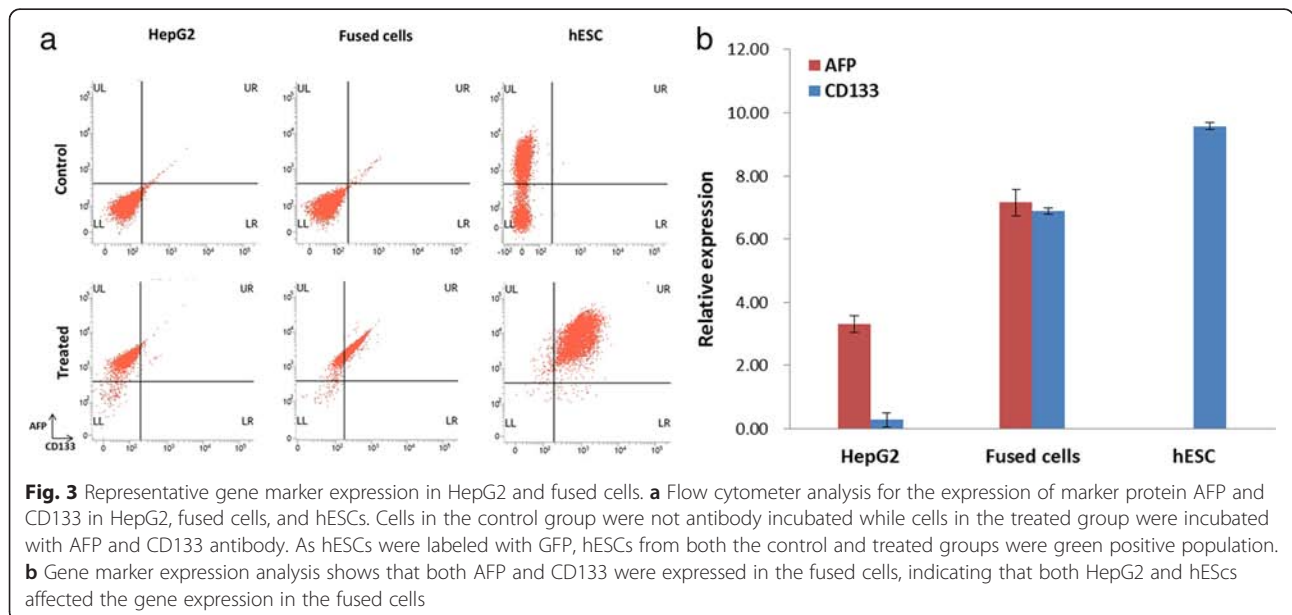
Before the fusion experiment, the single cells were obtained with the enzymatic treatment for passaging. After proper neutralization, the single-cell suspension in the culture medium was stored temporarily at 4 °C. To

distinguish hESCs from HepG2, hESCs were labeled with Oct-GFP and HepG2 cells were stained with red mitochondrion selective probe. For mitochondria staining, the HepG2 single cells were incubated in a 40 nM MitoTracker probe (Life Technology) for 15 min and washed three times. The stained cells were re-suspended in DMEM with 10 % FBS.

The fusion experiment was performed, as described in the literature [14]. One GFP labeled hESC and one mitochondria-stained HepG2 cell were trapped and manipulated using optical tweezers to form a cell pair. Laser scissors functioned at 10 Hz, and each pulse had a duration of 1 ns to cut the cell membrane at the point of contact between the two cells. Successful fusion was verified by observing the transfer of cytoplasmic GFP from the hESC to the HepG2 cell (as shown in Fig. 1). The fused cells attached to the cover glass bottom and survived.

**Cell isolation and colony formation**

A total of 24 h after fusion, the fused cells were collected following the trypsin-EDTA treatment and diluted in a low-density single-cell suspension (6 cells/mL to 10 cells/mL). In each well of a 96-well plate, 100 μL of the cell suspension was seeded. After 24 h of incubation, the plate was examined under a microscope, and the wells containing single cells were selected. Cells were fed with new DMEM medium every 2 days. After one week of culturing, colonies formed in the marked wells, and these colonies, which exhibited different morphologies than that of HepG2, were gradually replanted to 35 mm dishes for further characterization.



**Table 2** Significantly enriched GO terms in DEGs

Gene ontology term	Cluster frequency	Genome frequency of use	Corrected <i>p</i> -value
Immune response	10.2 %	4.9 %	2.74e-07
Immune system process	12.4 %	6.8 %	3.77e-06
Response to virus	2.6 %	0.8 %	0.00138
Regulation of apoptosis	7.8 %	4.3 %	0.00508
Regulation of programmed cell death	7.8 %	4.4 %	0.00840
Regulation of cell death	7.8 %	4.4 %	0.00888

### Gene sequencing and differential gene expression analysis

For each sample, the total RNA was sent to the BGI Company for RNA-Seq (Quantification) sequencing and screening of differentially expressed genes (DEGs).

### Flow cytometry

We performed FACS tests on HepG2, fused cells and hESCs, respectively. Cells were detached from culture dishes and suspended in 100  $\mu$ l of buffer (Miltenyi Biotec) and human serum (1:1) for 15 min at 4°C. Cells were then incubated for 45 min with a primary antibody for AFP (Santa Cruz Biotechnology, Cat No. sc-8399) and CD133-PE (Miltenyi Biotec, Cat No. 130-098-829). Cells were washed with FACS buffer, and incubated for 30 min at 4°C with a secondary antibody (Santa Cruz Biotechnology, Cat No. sc-2856). Cell labeling was detected using FACSVerse™ (BD Pharmingen). Flow cytometry results were analyzed by using BD FACSuite software.

### Quantitative RT-PCR

Total RNA was extracted from the cells with Trizol reagent (Invitrogen) using the method provided by the manufacturer. Reverse transcribed cDNA was produced

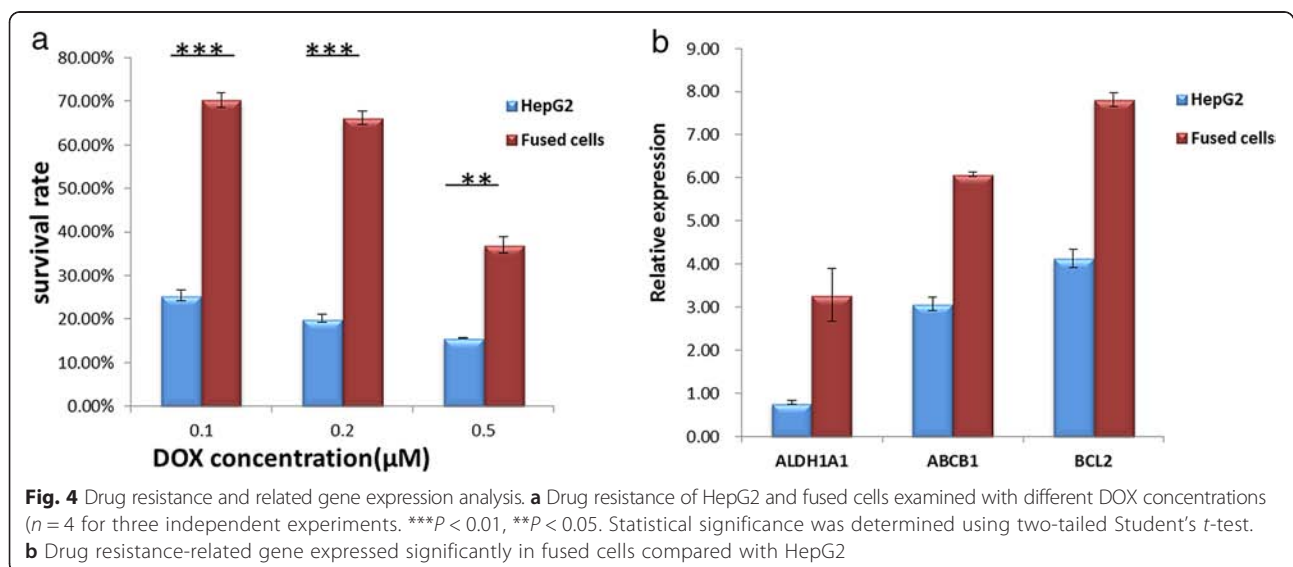
with iScript™ cDNA synthesis kit (Bio-Rad, Cat No170-8890). Real-time quantitative PCR amplification was performed with SsoAdvanced SYBR Green supermix kit (Bio-Rad, Cat No. 1725260) in CFX96 Real-time System (Bio-Rad, USA). The specific primers used in the analyses are listed in Table 1. The CD133 and CD44 primers were described in [15] and [16]. Additional primer sequences were obtained from the Primer Bank [17].

### Drug resistance

The fused cells and HepG2 were first incubated in a 6-well plate for 15 h. After this step, 0.1, 0.2, and 0.5  $\mu$ M DOX were added to the culture medium with three parallel samples for every group. The cells were incubated for an additional 48 h. Cell viability was determined through the trypan blue exclusion test.

### In vivo tumorigenicity assay

HepG2 cells and the fused cells ( $5 \times 10^4$ ,  $1 \times 10^5$ , and  $1 \times 10^6$  cells in 100  $\mu$ l DMEM) were subcutaneously injected into the right scapula of each nude mouse (BALB/c nu/nu, 4 to 6 weeks old). Four nude mice were prepared for each group. Tumor growth in the nude mice was measured every 7 days for 6 weeks. The nude mice were sacrificed at week 6 or 7. The tumors were



fixed with formaldehyde solution and stained with hematoxylin-eosin (H&E).

#### Animal care and ethics statement

Male athymic nude mice (BALB/c nu/nu, 4 to 6 weeks old) were used in this study. These animals were housed in pathogen-free conditions and provided with food and water at the facility of LKS Faculty of Medicine, University of Hong Kong (HKU). The Committee on the Use of Live Animals in Teaching and Research, HKU approved the protocol.

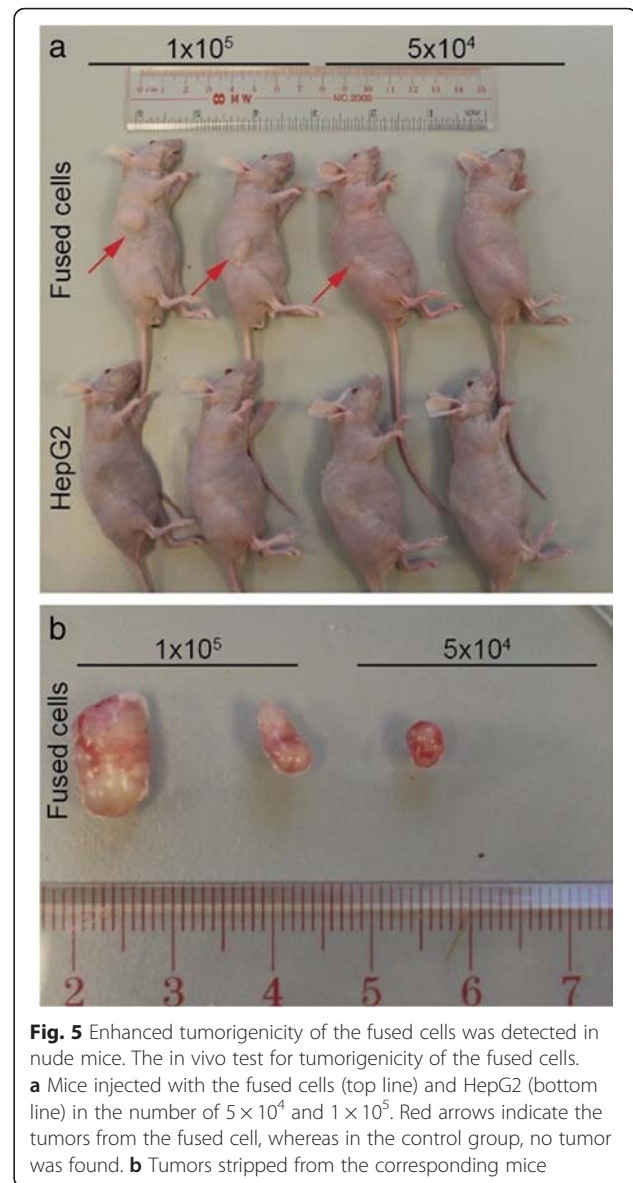
#### Statistical analysis

Data on RT-PCR results and drug resistance were expressed as mean  $\pm$  SD. Data from in vivo tumor experiment was expressed as mean  $\pm$  s.e.m. Statistical analysis was performed by adopting the two-tailed Student's *t*-test. Differences with  $p \leq 0.05$  were considered significant.

## Results

### Gene expression of the fused cells is different from HepG2 cells

As shown in Fig. 2, the isolated fused cells did not exhibit a conspicuous spindle shape, which was different from the morphology of HepG2 cells and hESC. The marker protein and gene expression of the fused cells and their parent cells were also detected, and the results showed that both HepG2 and hESCs affected the fused cells (see Fig. 3). To identify the functional difference between the fused cell and the HepG2 cells, we conducted pairwise comparisons to examine the significantly different molecular function, biological process, and cellular component (Gene Ontology [GO]). As indicated in Table 2, the immune response, immune system process, and regulation of apoptosis of the differential expression genes improved. Similar to CD133, CD44 exhibited a different expression in the fused cells and HepG2 cells in the analysis. We conducted qPCR tests for the related genes. The results showed that several liver tumor-initiating cell markers, including AFP, CD133, CD44, and EpCAM [18], were unregulated in the fused cells compared with those of HepG2 cells (Figs. 3b and 7). CD133 is an important stemness biomarker in normal stem cells and TICs, which can help maintain tumorigenic property and affect the tumor growth of the cells [19]. CD44 invariant regulates the redox status, which affects tumor initiation, tumorigenesis, and metastasis [20]. CD133 and CD44 are rarely expressed in normal cancer cells. EpCAM is a key protein that activates the Wnt signaling pathway, which is related to tumorigenesis, invasion, and resistance to drug treatments. The high expression of EpCAM enhances the chemoresistance of cells [21–23].



**Fig. 5** Enhanced tumorigenicity of the fused cells was detected in nude mice. The in vivo test for tumorigenicity of the fused cells. **a** Mice injected with the fused cells (top line) and HepG2 (bottom line) in the number of  $5 \times 10^4$  and  $1 \times 10^5$ . Red arrows indicate the tumors from the fused cell, whereas in the control group, no tumor was found. **b** Tumors stripped from the corresponding mice

**Table 3** In vivo tumor development experiments of HepG2 cells and fused cells in nude mice

Cell type	Cell numbers injected	Tumor incidence <sup>a</sup>	Latency (days) <sup>b</sup>
HepG2	$5 \times 10^4$	0/4	—
	$1 \times 10^5$	0/4	—
	$1 \times 10^6$	1/2	14
Fused cells	$5 \times 10^4$	2/4	14
	$1 \times 10^5$	3/4	14
	$1 \times 10^6$	4/4	12

<sup>a</sup>Number of tumors detected/number of injections

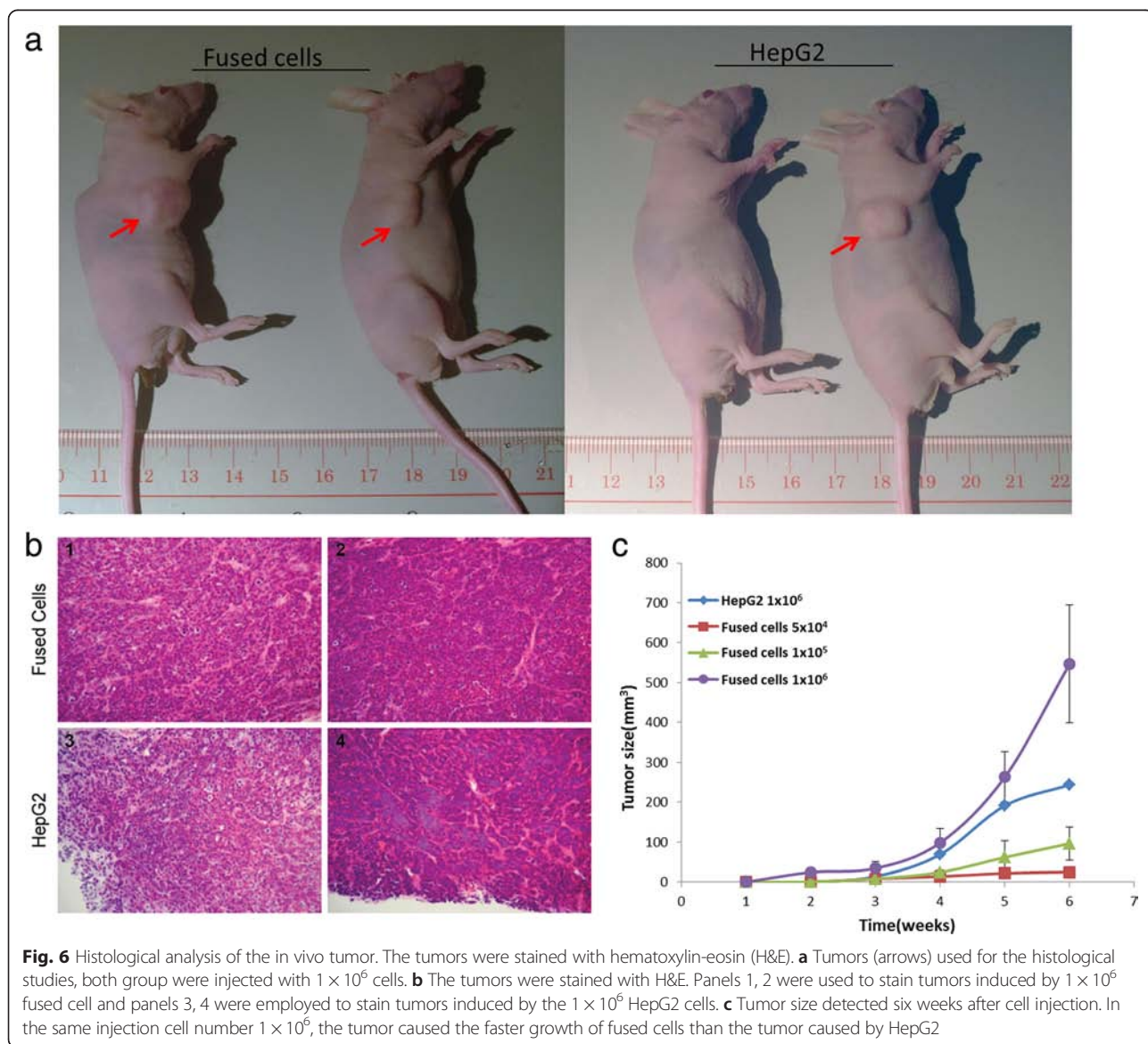
<sup>b</sup>Approximate number of days from tumor cell injection to appearance of a tumor

**Fused cells exhibited increased drug resistance**

Drug resistance of the fused cells was also examined. Fused cells and HepG2 were treated using different concentrations of doxorubicin (DOX) for 48 h. Figure 4a illustrates that the survival rate of the fused cell was significantly higher than that of HepG2 and the survival rates of the fused cell and HepG2 decreased as DOX concentration increased. In the present study, survival rate was defined as the number of viable cells after DOX treatments divided by the number of viable cells without DOX treatments. The results demonstrated that the fused cells exhibited increased drug resistance compared with their donor HepG2 cells.

The qPCR test results indicated that CD133 (Fig. 3b), ALDH1A1, ATP-binding cassette sub-family B member 1 (ABCB1), and B-cell lymphoma 2(Bcl-2) (Fig. 4b) were

highly expressed in the fused cells. High expression of CD133 is associated with drug resistance and relapses in a variety of solid tumors. ALDH is an effective detoxifying enzyme, and a high expression of ALDH can provide a route for tumors to resist chemotherapy. The expression of ABCB1 has the capacity of active DNA repair and resistance to apoptosis [24, 25]. The anti-apoptotic protein Bcl-2 has been proven to cause resistance to cancer treatment by forming heterodimers with a number of pro-apoptotic proteins [26, 27]. Bcl-2 also contributes to the resistance to a wide spectrum of chemotherapeutic agents [28, 29]. The expression of CD133, ABCB1, Bcl-2, and ALDH1A1 in the fused cells explains the increased drug resistance of the fused cell [30], suggesting that the cancer cell would acquire drug resistance through cell fusion with the stem cell.



**Fig. 6** Histological analysis of the in vivo tumor. The tumors were stained with hematoxylin-eosin (H&E). **a** Tumors (arrows) used for the histological studies, both group were injected with  $1 \times 10^6$  cells. **b** The tumors were stained with H&E. Panels 1, 2 were used to stain tumors induced by  $1 \times 10^6$  fused cell and panels 3, 4 were employed to stain tumors induced by the  $1 \times 10^6$  HepG2 cells. **c** Tumor size detected six weeks after cell injection. In the same injection cell number  $1 \times 10^6$ , the tumor caused the faster growth of fused cells than the tumor caused by HepG2

### Fused cells are highly tumorigenic

To analyze tumor-initiating capability, nude mice were transplanted with various amounts of the fused cells. The same amounts of HepG2 cells were used as the control. The tumor volume was measured weekly. The fused cells could result in progressively growing tumors in the nude mice with fewer cells compared with HepG2 cells. The injection of fused cells at  $5 \times 10^4$  facilitated the progressive growth of tumors in the nude mice, whereas the injection of HepG2 cells at  $5 \times 10^4$  did not induce this effect (Fig. 5). Tumor incidence increased when the injection dose of the fused cells was raised to  $1 \times 10^5$ . However, tumors were still not formed in the mice injected with the same amount of HepG2 cells. When the injected cells were increased to  $1 \times 10^6$ , both the fused and HepG2 cells promoted tumor growth in the nude mice. The fused cells induced 100 % tumor incidence, whereas the HepG2 cells induced only 50 % tumor incidence. The fused cell possessed higher tumorigenicity and faster tumor growth than the HepG2 cells (Table 3 and Fig. 6c).

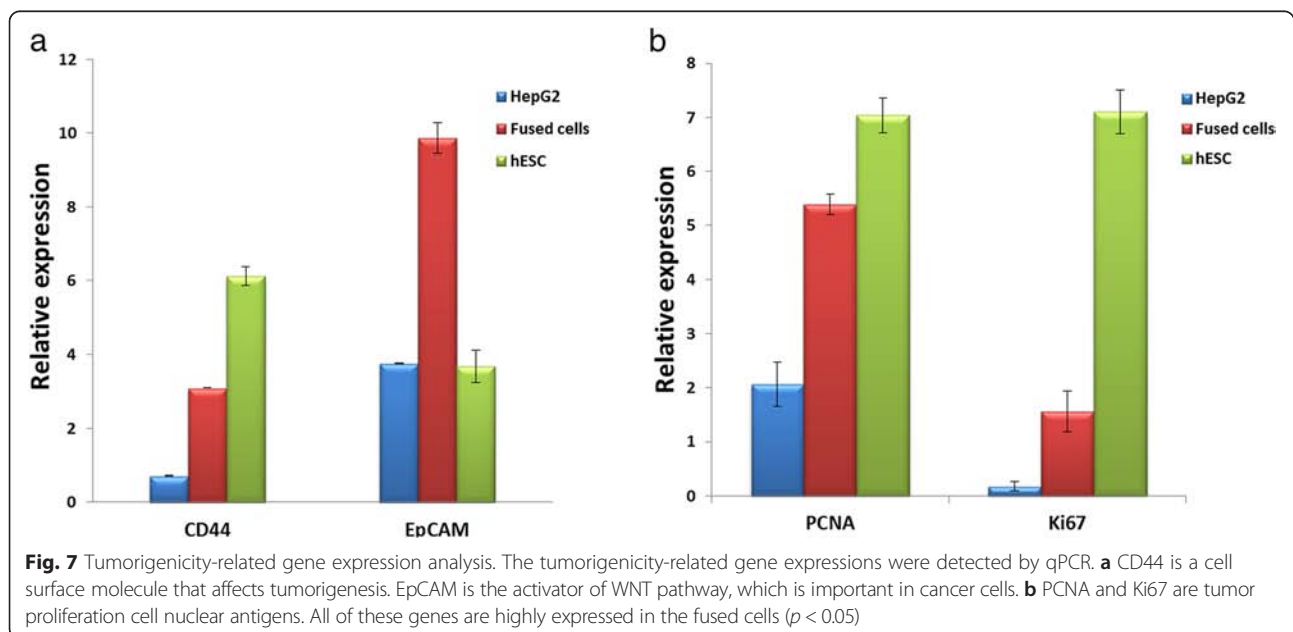
As shown in Fig. 5a and b, the xenograft tumors exhibited similar histological features in the mice injected with the fused cells or HepG2 cells, which indicated that the tumor tissue was not a teratoma induced by hESCs. The result of *in vivo* tumorigenicity assay showed that the fused cells exhibited increased tumorigenicity *in vivo*. The highly expressed CD133 (Fig. 3), CD44, and EpCAM may illustrate the mechanism of enhanced tumorigenicity (Fig. 7a). The expression of two tumor proliferation factors, proliferating cell nuclear antigen (PCNA) and ki67 were also detected. The qPCR results indicated that both PCNA and Ki67 were expressed

relatively high in the fused cell (Fig. 7b). The high expression of PCNA indicates the high risk of carcinogenesis [31]. Ki67 is a nuclear antigen expressed in a variety of solid malignant tumors, and its expression level is significantly higher than that of normal tissue. Ki67 is closely related to the development, metastasis, and prognosis of malignant tumors [32, 33].

### Discussions

Using engineered laser-induced single-cell fusion technology, we have successfully produced a fused cell line, which coalesces the gene expression information of hepatocellular carcinoma cells and stem cells. Experimental results verified that the fused cells are highly tumorigenic and chemoresistant compared with the donor hepatocellular carcinoma cells. The fusion of stem cells and cancer cells enables the hybrids to exhibit stemness and cancer characteristics. Traditionally, during stem cell induced reprogramming of somatic cell via fusion, the somatic pluripotency gene epigenetic modifications has been recognized to be a crucial factor, which has been explained as one of the parental cells dominating phenotype over the other [34]. The epigenetic modifications include chromatin remodeling, histone modification and DNA demethylation [35, 36]. However, the mechanism of fusion between cancer cells and stem cells are rarely elaborated.

Our study on artificially engineered fusion of stem cells and liver cancer cells demonstrated a possible mechanism of the tumor-initiating cell generation in the human body. The fusion of stem cells can occur spontaneously *in vivo* after stem cell injection or transplant [37]; thus, our research may explain the risk induced by





stem cell therapy in liver cancer. On one hand, some stem cell therapies may generate cancer stem-like cells and exacerbate cancer instead of curing the disease. On the other hand, the artificial generation of tumor initiating-like cells can be used to produce malignant cells for cancer stem cell drug screening and studies on its mechanism.

## Conclusions

We artificially fused a HepG2-hESC cell pair and isolated a fused cell line. The genetic analysis result demonstrated that the fusion of cancer cells with stem cells resulted in cancer cells that were more tumorigenic and exhibited higher chemoresistance. The result also indicated that the fused cells were highly similar to tumor-initiating cells in biological processes or cellular compartments. The related gene expressions, such as CD133, CD44, and EpCAM, in the fused cells also supported this conclusion. In the future work, we will continue to explore the stem cell mechanism (e.g., functional protein assay and GO comparison to original TICs), as well as conduct more sufficient *in vivo* experimental study.

## Abbreviations

hESC: human embryonic stem cell; HCC: hepatocellular carcinoma; TSCs: tumor stem cells; TICs: tumor-initiating cell; DOX: doxorubicin; MOI: multiplicity of infection; H&E: hematoxylin-eosin; ALDH1A1: aldehyde dehydrogenase 1 family, member A1; ABCB1: ATP-binding cassette sub-family B member 1; Bcl-2: B-cell lymphoma 2; PCNA: proliferating cell nuclear antigen.

## Competing interests

The authors declare that they have no competing interests.

## Authors' contributions

DS and KM developed the concept and worked with SHC, RL and CML to conceive the project. RW, SC, KM, and DS designed the study, analyzed the data and wrote the paper. RW, SC, CL, and TPN conducted the experiments. SHC provided human liver hepatocellular carcinoma cells, RL, MK provided human embryonic stem cells, besides providing advises to cell biology studies. CML and JC contributed to the data analysis. All authors read the manuscript and agreed with the presentation of its contents.

## Acknowledgment

This work was supported by grants from the Research Grants Council of the Hong Kong Special Administrative Region, China (Reference No. CityU9/CRF/13G and CityU 11210315) and the Theme-based Research Scheme (T13-706).

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Received: 10 September 2015 Accepted: 29 January 2016



## References

- Ficz G, Reik W. Reprogramming by cell fusion: boosted by Tets. *Mol Cell*. 2013;49(6):1017–8. doi:10.1016/j.molcel.2013.03.014.

- Ferrand J, Noel D, Lehours P, Prochazkova-Carlotti M, Chambonnier L, Menard A, et al. Human bone marrow-derived stem cells acquire epithelial characteristics through fusion with gastrointestinal epithelial cells. *PLoS One*. 2011;6(5):e19569. doi:10.1371/journal.pone.0019569.
- Piccolo FM, Bagci H, Brown KE, Landeira D, Soza-Ried J, Feytout A, et al. Different roles for Tet1 and Tet2 proteins in reprogramming-mediated erasure of imprints induced by EGC fusion. *Mol Cell*. 2013;49(6):1023–33. doi:10.1016/j.molcel.2013.01.032.
- Yamanaka S, Blau HM. Nuclear reprogramming to a pluripotent state by three approaches. *Nature*. 2010;465(7299):704–12. doi:10.1038/Nature09229.
- Forbes SJ, Newsome PN. New horizons for stem cell therapy in liver disease. *J Hepatol*. 2012;56(2):496–9. doi:10.1016/j.jhep.2011.06.022.
- Sakaida I, Terai S, Yamamoto N, Aoyama K, Ishikawa T, Nishina H, et al. Transplantation of bone marrow cells reduces CCl4-induced liver fibrosis in mice. *Hepatology*. 2004;40(6):1304–11. doi:10.1002/hep.20452.
- Furst G, Schulte am Esch J, Poll LW, Hosch SB, Fritz LB, Klein M, et al. Portal vein embolization and autologous CD133+ bone marrow stem cells for liver regeneration: initial experience. *Radiology*. 2007;243(1):171–9. doi:10.1148/radiol.2431060625.
- Newsome PN, Johannessen I, Boyle S, Dalakas E, McAulay KA, Samuel K, et al. Human cord blood-derived cells can differentiate into hepatocytes in the mouse liver with no evidence of cellular fusion. *Gastroenterology*. 2003;124(7):1891–900. doi:10.1016/S0016-5085(03)00401-3.
- Visvader JE, Lindeman GJ. Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. *Nat Rev Cancer*. 2008;8(10):755–68. doi:10.1038/nrc2499.
- Dang H, Ding W, Emerson D, Rountree CB. Snail1 induces epithelial-to-mesenchymal transition and tumor initiating stem cell characteristics. *BMC Cancer*. 2011;11:396. doi:10.1186/1471-2407-11-396.
- Yamashita T, Wang XW. Cancer stem cells in the development of liver cancer. *J Clin Invest*. 2013;123(5):1911–8. doi:10.1172/JCI66024.
- Yamashita T, Kaneko S. Orchestration of hepatocellular carcinoma development by diverse liver cancer stem cells. *J Gastroenterol*. 2014;49(7):1105–10. doi:10.1007/s00535-014-0951-1.
- Zhang F, Chen XP, Zhang W, Dong HH, Xiang S, Zhang WG, et al. Combined hepatocellular cholangiocarcinoma originating from hepatic progenitor cells: immunohistochemical and double-fluorescence immunostaining evidence. *Histopathology*. 2008;52(2):224–32. doi:10.1111/j.1365-2559.2007.02929.x.
- Chen SX, Cheng JP, Kong CW, Wang XL, Cheng SH, Li RA, et al. Laser-induced fusion of human embryonic stem cells with optical tweezers. *Appl Phys Lett*. 2013;103(3):033701. doi:10.1063/1.4813822.
- Wagner W, Ansorge A, Wirkner U, Eckstein V, Schwager C, Blake J, et al. Molecular evidence for stem cell function of the slow-dividing fraction among human hematopoietic progenitor cells by genome-wide analysis. *Blood*. 2004;104(3):675–86. doi:10.1182/blood-2003-10-3423.
- Mima K, Okabe H, Ishimoto T, Hayashi H, Nakagawa S, Kuroki H, et al. CD44s regulates the TGF-beta-mediated mesenchymal phenotype and is associated with poor prognosis in patients with hepatocellular carcinoma. *Cancer Res*. 2012;72(13):3414–23. doi:10.1158/0008-5472.CAN-12-0299.
- Wang XW, Seed B. A PCR primer bank for quantitative gene expression analysis. *Nucleic Acids Res*. 2003;31(24):e154. doi:10.1093/nar/gng154.
- Yamashita T, Wang XW. Cancer stem cells in the development of liver cancer. *J Clin Invest*. 2013;123(5):1911–8. doi:10.1172/JCI66024.
- Yao J, Zhang T, Ren J, Yu M, Wu G. Effect of CD133/prominin-1 antisense oligodeoxynucleotide on *in vitro* growth characteristics of Huh-7 human hepatocarcinoma cells and U251 human glioma cells. *Oncol Rep*. 2009;22(4):781–7.
- Patrawala L, Calhoun-Davis T, Schneider-Broussard R, Tang DG. Hierarchical organization of prostate cancer cells in xenograft tumors: The CD44(+)alpha 2 beta 1(+) cell population is enriched in tumor-initiating cells. *Cancer Res*. 2007;67(14):6796–805. doi:10.1158/0008-5472.Can-07-0490.
- Yamashita T, Ji J, Budhu A, Forgues M, Yang W, Wang HY, et al. EpCAM-positive hepatocellular carcinoma cells are tumor-initiating cells with stem/progenitor cell features. *Gastroenterology*. 2009;136(3):1012–24. doi:10.1053/j.gastro.2008.12.004.
- Ji JF, Yamashita T, Budhu A, Forgues M, Jia HL, Li CL, et al. Identification of MicroRNA-181 by genome-wide screening as a critical player in EpCAM-positive hepatic cancer stem cells. *Hepatology*. 2009;50(2):472–80. doi:10.1002/Hep.22989.

23. Gostner JM, Fong D, Wrulich OA, Lehne F, Zitt M, Hermann M, et al. Effects of EpCAM overexpression on human breast cancer cell lines. *BMC Cancer*. 2011;11:45. doi:10.1186/1471-2407-11-45.
24. Dean M, Fojo T, Bates S. Tumour stem cells and drug resistance. *Nat Rev Cancer*. 2005;5(4):275–84. doi:10.1038/nrc1590.
25. Houthuijzen JM, Daenen LG, Roodhart JM, Voest EE. The role of mesenchymal stem cells in anti-cancer drug resistance and tumour progression. *Br J Cancer*. 2012;106(12):1901–6. doi:10.1038/bjc.2012.201.
26. Abdullah LN, Chow EK. Mechanisms of chemoresistance in cancer stem cells. *Clin Translat Med*. 2013;2(1):3. doi:10.1186/2001-1326-2-3.
27. Enyedy IJ, Ling Y, Nacro K, Tomita Y, Wu XH, Cao YY, et al. Discovery of small-molecule inhibitors of bcl-2 through structure-based computer screening. *J Med Chem*. 2001;44(25):4313–24. doi:10.1021/Jm010016f.
28. van Oosterwijk JG, Herpers B, Meijer D, Briaire-de Bruijn IH, Cleton-Jansen AM, Gelderblom H, et al. Restoration of chemosensitivity for doxorubicin and cisplatin in chondrosarcoma in vitro: BCL-2 family members cause chemoresistance. *Ann Oncol*. 2012;23(6):1617. doi:10.1093/annonc/mdr512.
29. Siddique HR, Paray A, Tarapore RS, Wang L, Mukhtar H, Karnes RJ, et al. BMI1 polycomb group protein acts as a master switch for growth and death of tumor cells: regulates TCF4-transcriptional factor-induced BCL2 signaling. *PLoS One*. 2013;8(5):e60664. doi:10.1371/journal.pone.0060664.
30. Ji J, Wang XW. Clinical implications of cancer stem cell biology in hepatocellular carcinoma. *Semin Oncol*. 2012;39(4):461–72. doi:10.1053/j.seminoncol.2012.05.011.
31. Kobayashi I, Matsuo K, Ishibashi Y, Kanda S, Sakai H. The proliferative activity in dysplasia and carcinoma in situ of the uterine cervix analyzed by proliferating cell nuclear antigen immunostaining and silver-binding argyrophilic nucleolar organizer region staining. *Hum Pathol*. 1994;25(2):198–202.
32. Inwald EC, Klinkhammer-Schalke M, Hofstadter F, Zeman F, Koller M, Gerstenhauer M, et al. Ki-67 is a prognostic parameter in breast cancer patients: results of a large population-based cohort of a cancer registry. *Breast Cancer Res Tr*. 2013;139(2):539–52. doi:10.1007/s10549-013-2560-8.
33. Luporsi E, Andre F, Spyrtos F, Martin PM, Jacquemier J, Penault-Llorca F, et al. Ki-67: level of evidence and methodological considerations for its role in the clinical management of breast cancer: analytical and critical review. *Breast Cancer Res Tr*. 2012;132(3):895–915. doi:10.1007/s10549-011-1837-z.
34. Lluís F, Cosma MP. Cell-fusion-mediated somatic-cell reprogramming: a mechanism for tissue regeneration. *J Cell Physiol*. 2010;223(1):6–13. doi:10.1002/Jcp.22003.
35. Silva J, Chambers I, Pollard S, Smith A. Nanog promotes transfer of pluripotency after cell fusion. *Nature*. 2006;441(7096):997–1001. doi:10.1038/nature04914.
36. Wong CC, Gaspar-Maia A, Ramalho-Santos M, Reijo Pera RA. High-efficiency stem cell fusion-mediated assay reveals Sall4 as an enhancer of reprogramming. *Plos One*. 2008;3(4):e1955. doi:10.1371/journal.pone.0001955.
37. Cusulin C, Monni E, Ahlenius H, Wood J, Brune JC, Lindvall O, et al. Embryonic stem cell-derived neural stem cells fuse with microglia and mature neurons. *Stem Cells*. 2012;30(12):2657–71. doi:10.1002/Stem.1227.

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