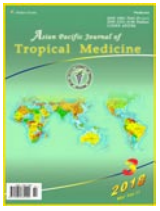

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Effects of regenerated tissue extracts after liver injury on the proliferation, differentiation, migration and invasion of SK-HEP1 cells

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ABSTRACT

Objective: To study the effects of regenerated tissue extracts after liver injury on the proliferation, differentiation, migration and invasion of SK-HEP1 cells. **Methods:** Regenerated tissue extracts after liver injury were used to induce SK-HEP1 cells after enrichment, their effects on the proliferation, differentiation, migration and invasion of SK-HEP1 cells were observed through *in vitro* cell culture, MTT, flow cytometry and transwell assays. **Results:** In response to the action of regenerated tissue extracts after liver injury, SK-HEP1 cells were blocked in G_0/G_1 phase, their growth rate was distinctly reduced. The number of SK-HEP1^{-fj} colonies decreased. The migration ability of SK-HEP1 cells showed a decreased trend on day 7 and day 11 after induction. SK-HEP1's invasion ability clearly decreased on days 7 and 11 after induction, especially on day 7. **Conclusions:** To a certain extent, regenerated tissue extracts after liver injury can inhibit the proliferation, differentiation, migration and invasion of hepatoma cells, showing an important potential of being a differentiating agent for the treatment of liver cancer.

1. Introduction

Hepatocellular carcinoma (HCC) is the most common histological type of primary liver cancer, accounting for more than 90% of cases with an extremely high mortality rate[1]. With the increasing early detection rate of liver cancer, the improvements in liver resection and the application of liver transplantation, the long-term survival rate of patients with liver cancer has increased. However, there are still many problems, such as tumor chemotherapy resistance, metastasis and recurrence, and dyscrasia.

Differentiation-inducing therapy is a new method of tumor treatment[2] that has been applied to the treatment of clinical hematologic malignancies in recent years, with around a 90% remission rate[3]. The majority of experts believe that the main cause of these malignancies is cancer stem cells[4]. Due to the different differentiation levels of tumor tissue, a variety of cell types are present, including cancer stem cells/tumor-initiating cells

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(CSCs/TICs), transitional cells, and terminally differentiated cells[5]. Although CSCs only occupy a small proportion of the tumor tissue, their features of infinite proliferation and differentiation result in continuous tumor recurrence and metastasis. Only by controlling these CSCs it would be possible to control cancer recurrence and metastasis[6-8]. It is still not clear whether it is possible to 'transform' cancer cells (especially cancer stem cells) and block them in a 'dormant period' by using differentiation-inducing therapy, and thus, we designed this research to discuss the effects of regenerated tissue extracts after liver injury on the proliferation, differentiation, migration and invasion of SK-HEP1 cells.

2. Materials and methods

2.1. Cells

The human hepatoma cell line SK-HEP1 was provided by the Pathology Laboratory of the First Affiliated Hospital of Sun Yat-sen University and Sun Yat-sen University Cancer Center. The cells were referred to as SK-HEP1^{-fj} after enrichment.

2.2. Preparation of regenerated tissue extracts after liver injury

The Experimental Animal Center of Sun Yat-sen University provided clean-grade female Sprague-Dawley (SD) rats, aged 6 weeks and weighing 130–150 g. The research methods were according to previous literature about a rat model of 2-AAF/CCl_4 liver regeneration[9]. First, rats were sacrificed by cervical dislocation; then, the intact livers were removed and rinsed with sterile PBS. After that, the clean livers were placed in a petri dish (keep on ice) and cut into small pieces by using ophthalmic scissors. Then, small pieces were put into a homogenizer along with DMEM/F12 basal medium and ground repeatedly until a homogenate was formed. The next step was separation using high-speed and low-temperature EP centrifugation (1 3000 r/min, 4 ℃, 30 min) three times, and each supernatant was collected and centrifuged again. After three centrifugations, a 0.22 μm needle filter was used to filter the supernatant.

2.3. Cell culture

Preparation steps for DMEM/F12/RPIM1640 basal medium are as follows: DMEM/F12 powder (GIBCO, USA) was dissolved in tri-distilled water, and 1.2 g $NaHCO_3$ was added according to the instructions to adjust the pH to 7.2 (pH meter WTWpH522, Germany). The total volume of 1 000 mL medium was filtered and sterilized (using a filter sterilizer from the Corning company), then it was preserved at 4 ℃ for sterile experiments. The preparation method for RPIM1640 basal medium was similar to the above, the only difference was adding a different amount of $NaHCO_3$ (2 g).

To prepare DMEM/F12/RPIM1640 complete medium, a total of 10 mL L-glutamine (200 mM) was added into the above basic medium; then 10 mL superfine fetal bovine serum (TBD, Tianjin) per 100 mL basal medium was added; epidermal growth factor (EGF, PeproTech, UK) was also added to a final concentration of 10 ng/mL.

The hepatoma cells SK-HEP1 were inoculated into RPMI-1640 complete medium (10% fetal bovine serum, 100 U/L penicillin and 100 μg/L streptomycin) and incubating in the incubator (37 ℃, 5% CO_2).

2.4. Enrichment of SK-HEP1 cells

Cancer stem cells were enriched. When the cells' growth reached the logarithmic phase, 2 μg/mL DDP was added. In the logarithmic growth phase, 1.5×10^5 cells were taken respectively from each bottle and were divided into an induction and non-induction group. After 24 h, the induction group was put into conditioned medium with 0.15 g/L regenerated tissue extracts after liver injury, and the cells were collected on 0, 7, 11, 21 d for further research[10,11].

2.5. Quantitative analysis of $CD133^+/CD90^+$ cell populations

Cells were collected at different time points and washed twice with PBS. A total of 10^5 cells were stained with CD90-PC and CD133-PE antibodies [murine IgG1-PC5 and murine anti-human CD90-PC5, PHARMINGER USA; murine IgG2b-PE and murine anti-human CD133-PE, FcR blocker (MiltenyiBiotec)] were then added to the above cell suspension. They were incubated at room temperature for 20 min, washed with PBS, and detected by flow cytometry (Beckman-Coulter-Elite, USA)[12,13].

2.6. Differentiation-inducing experiment

The hepatoma cells SK-HEP1 (after enrichment) were inoculated into complete medium (containing 10% fetal bovine serum, 100 U/L penicillin and 100 μg/L streptomycin) and cultured in an incubator (37 ℃, 5% CO_2). The cells were then inoculated into conditioned medium (containing 0.15 mg/mL regenerated tissue extracts after liver injury) after 24 h. Cells were collected at day 0, 7, 11 and 21 of culture for further experiments.

2.7. Cell cycle assessment

Cells at different time points (after enrichment) were collected and washed twice with PBS, then fixed in 70% cold ethanol (4℃) overnight. After that, the cells were washed with PBS another 2 times; the supernatant was removed, and DNA dye was added (containing iodide PI and RNA enzymes); the cell cycle was detected by flow cytometry after 30 min at room temperature (EPICS ALTRA,Beckman-Coulter company).

2.8. Cell proliferation assessment

MTT assays were used to detect the extracts' effects on the growth of SK-HEP1 (after enrichment) stem cells. Collected cells were resuspended in basal medium and added into 96 well plates (200 μL/well, with cell density of 3 000 cells/well). Cell-free wells were filled with sterile PBS. Then, they were cultured in an incubator (5% CO_2, 37 ℃) for 24 h, with the addition of tissue extracts after liver injury. A total of 20 μL/well of MTT solution (5 g/L) was added and incubation was continued for a further 4 h. After that, the medium was removed, and 150 μL/well DMSO was added instead, shaken

for 10 min at a low speed. The absorbance value of each well was measured at A490 nm using a microplate reader, and the growth curve of the cells was observed on day 6.

2.9. Analysis of differentiation ability

After collecting cells at various time intervals, counting and resuspending, an equal number of SK-HEP1 cells (500 cells/5 mL) were inoculated into 6 cm culture dishes and placed in a 37 ℃ incubator. Cell clones were observed after 14 d (cell population> 50 cells), and the culture medium was discarded, fixing with methanol for 30 min. Then, they were washed with PBS and stained with Gimesa working solution for 15–30 min. After that, they were washed with slow running water, dried out and the number of cell colonies was observed under a microscope.

2.10. Analysis of migration and invasion ability

The total amount of 5000 SK-HEP1 cells (after enrichment) was added to the upper chamber (basal medium) in transwell experiments, and complete medium containing 10% FBS was added to the lower chamber. The upper chamber was taken out after 24 h and fixed in 75% ethanol and stained with crystal violet. The amount of cells entering into the lower chamber was observed under the microscope. The procedure for detecting the invasion was similar except that matrigel was applied to the upper chamber instead.

2.11. Compliance with ethical standards

Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of Sun Yat-sen University Ethics Committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent: Informed was obtained from all individual participants included in the study.

2.12. Statistical analysis

All statistical analyses were performed using SPSS version 22 for Windows (SPSS Inc., Chicago, IL, USA). Data are presented as mean ± SD. For pair-comparisons, we applied a two-tailed paired *t*-test. A P value of < 0.05 was considered statistically significant.

3. Results

Under the action of regenerated tissue extracts of liver injury, it was clear that the growth rate of SK-HEP1^{-fj} cells slowed down and their cell viability decreased accompanied by various degrees of morphological changes. The MTT results also showed that regenerated tissue extracts of liver could inhibit the growth of hepatoma cells (Figure 1).

Compared with pre-induction, the proportion of SK-HEP1^{-fj} cells (induced for 21 d) in G_0/G_1 phase increased significantly, and the proportion in S phase decreased significantly. The decrease of proliferation index PI resulted in more hepatoma cells blocked in G_0/G_1 phase (Figure 2). Cell clones (Figure 3) were reduced on 7 and 11 d after induction, especially on 7 d. The number of $CD133^+CD90^+$ cells decreased on day 7, 11 and 21 after induction (Figure 4). Migration (Figure 5) and invasion (Figure 6) ability of SK-HEP1^{-fj} cells declined on 7 and 11 d after induction and then rebounded upwards by day 21. It was concluded that regenerated tissue extracts after liver injury inhibited the proliferation, differentiation, migration and invasive abilities of SK-HEP1 cells.

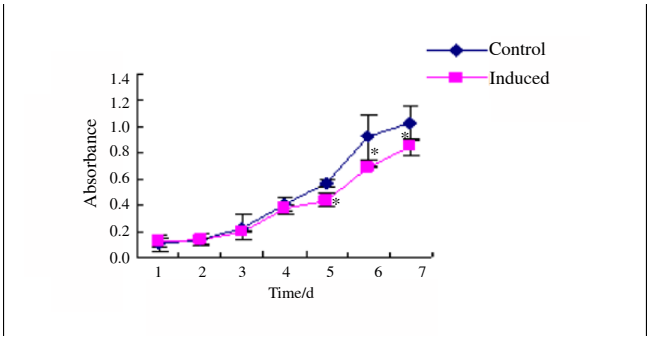

Figure 1. Comparison of the SK-HEP1^{-fj} cell growth curve between the induced and non-induced group (OD_{490nm}).

Compared with the non-induced group, the slope of the cell growth curve of the induced group clearly decreased after the fifth day. The results show that the induction of cell activity was inhibited and the growth rate slowed down. After 5 d, there was statistically significance between the induced and non-induced group. (P<0.05).

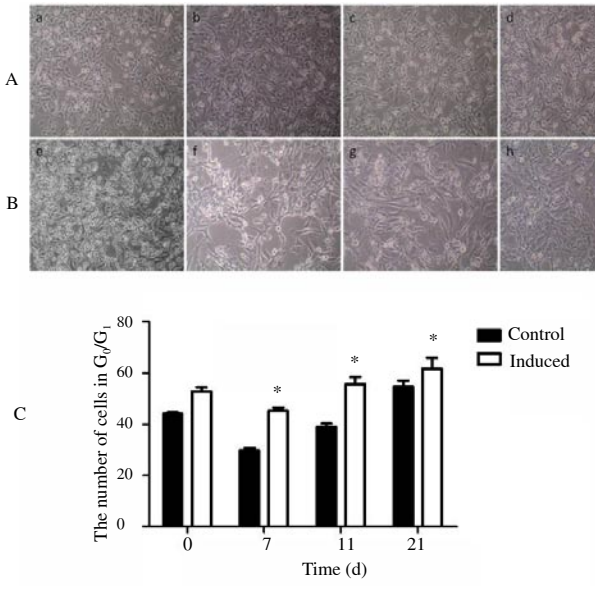

Figure 2. Comparison of cell morphology (×10) and cell cycle G_0/G_1 distribution between the induced and non-induced group.

(A) Cell morphology under the microscope at 0, 7, 11 and 21 d. The non-induced group showed cell growth and strong vitality, the mitotic images are more visible and there is pathological mitosis; (B) Cell morphology under the microscope at 0, 7, 11 and 21 d. The morphological changes in the induced group were not significant; however, the cell growth rate was significantly slowed down, the number of mitotic figures decreased, and part of the nuclear cytoplasm decreased; (C) The G_0/G_1 quantification of the cell cycle in the induction and non-induction groups were different at different time points ($^*P$<0.05).

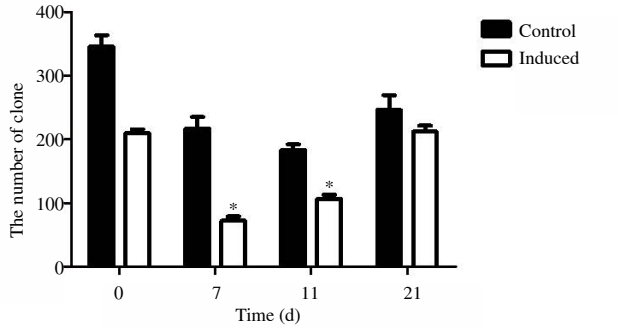

Figure 3. Comparison of cell cloning ability between the induced and non-induced group.
There were statistically significant differences between the induced and non-induced group cell colonies at the same time points except for day 21 ($^{*}P<0.05$).

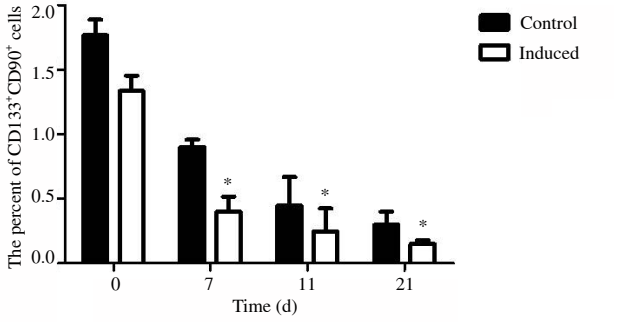

Figure 4. Comparison of the number of $CD133^{+}/CD90^{+}$ cells between the induced and non-induced group.
There were statistically significant differences between the induced and non-induced groups at the same time points ($^{*}P<0.05$).

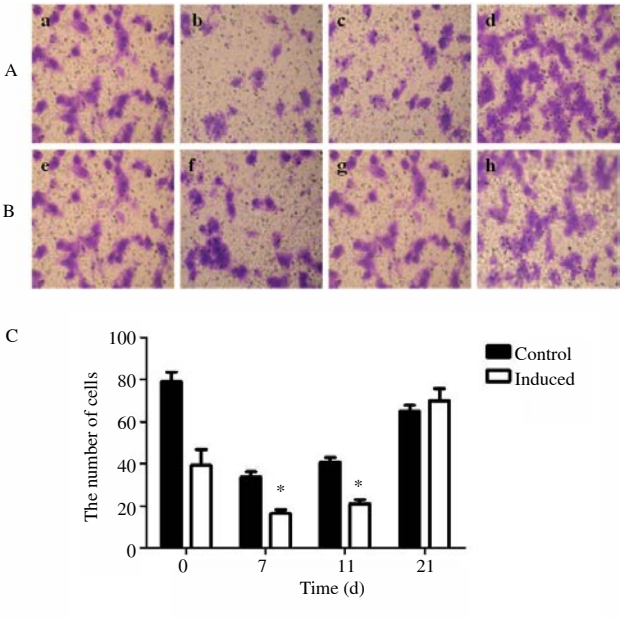

Figure 5. Comparison of migration of cells between the induced and non-induced group.
(A) The number of migrating cells in the induction group at different time points, a: 0 d; b: 7 d; c:11 d; d: 21 d; (B) The number of migrating cells in the non-induction group at different time points, a: 0 d; b:7 d; c: 11 d; d:21 d; (C) The number of migrating cells in the induced and non-induced group at the same time points were significantly different except for day 21 ($^{*}P<0.05$).

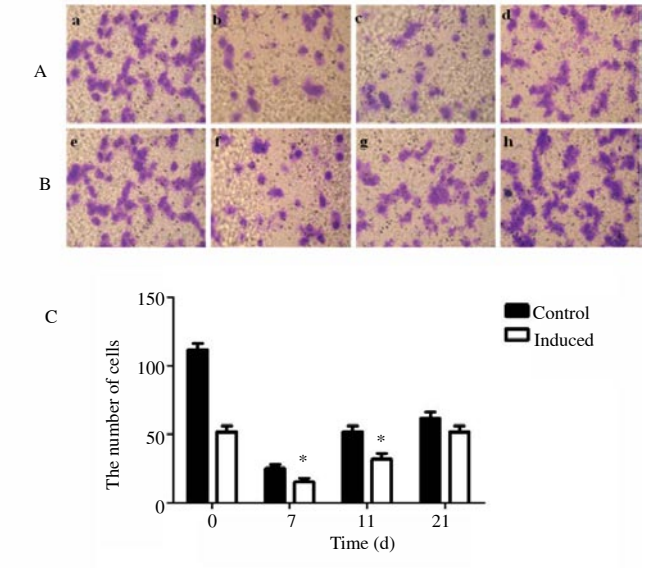

Figure 6. Comparison of invasive cell numbers between the induced and non-induced group.
(A) The number of invasive cells in the induction group at different time points, a: 0 d; b: 7 d; c: 11 d; d: 21 d; (B) The number of invasive cells in the non-induction group at different time points, a: 0 d; b: 7 d; c: 11 d; d: 21 d; (C) The number of invasive cells inthe induced and non-induced group at the same time points were significantly different except at day 21 ($^{*}P<0.05$).

4. Discussion

The concept of differentiation therapy was first proposed by Sachs in 1978[14]. In 1980, Breitman *et al.*[15] used retinoic acid to induce cellular differentiation of human promyelocytic leukemia HL-60. In 1988, a significant effect of all-trans retinoic acid (ATRA) application in inducing the differentiation of acute promyelocytic leukemia (APL) was achieved, with a complete remission rate of 90% in the clinic[3]. Differentiation-inducing therapy has been attracting increasing attentions. In recent years, with the establishment of solid tumor *in vitro* tumor cell lines, differentiation-inducing research has made some progress. Concerning nervous system tumors, Airoldi *et al.*[16] used low doses of interferon-γ (IFN-γ) to induce neuroblastoma cells to reduce their proliferation rate, and Oehme *et al.*[17] used histone deacetylases (HDACs) to induce the growth and differentiation of neuroblastoma cells. An HDAC inhibitor, 4-phenylbutyrate (4-PB), was used on pleomorphic glioma to slow down tumor proliferation[18]. Liu *et al.*[19] induced C6 cells to stay in the G_0/G_1 phase by inhibiting the protein synthesis of C6 glioma cells with low concentrations of cycloheximide. All of these results showed that the growth and proliferation rate of tumor cells could be changed by using corresponding inducers, and thus tumor treatment might be achieved.

Under the influence of regenerated tissue extracts after liver injury, the rate of growth was reduced after 5 d. The cell cycle of hepatoma cells had changed significantly. According to flow cytometry results, the proportion of SK-HEP1^{-fj} cells in G_0/G_1 phase after 7, 11, and 21 d induction increased significantly, and then the PI proliferation index decreased significantly. With the ability to induce cell proliferation, regenerated tissue extracts of liver injury can block more hepatoma cells in G_0/G_1 phase. Compared with the pre-induction, SK-HEP1^{-fj} induced at 7 d, 11 d had a reduced number of clones but not at 21 d; in particular, on day 7, there was

the most obvious reduction. The number of $CD133^{+}CD90^{+}$ cells decreased on day 7, 11 and 21 after SK-HEP1^{-fj} induction, especially on day 11. Further study is needed about complicated changes of various positive cells under the action of liver regeneration tissue extract such as on SK-HEP1^{-fj}, and this may be related to the cell characteristics and the extracts' sensitivity. The migration ability of SK-HEP1^{-fj} was decreased on the 7th and the 11th day, but the migration ability rebounded on the 21st day. The invasion ability of SK-HEP1^{-fj} decreased on the 7th and 11th day, especially on the 7th day. These results suggest that liver regeneration tissue extracts can reduce the migration and invasion of cells and clonality, and to a certain extent, reduce the tumor characteristics of HCC, showing potential as an inducer of differentiation.

The major limitation of the present study is that we only researched the effects of regenerated tissue extracts after liver injury from a cellular function aspect; thus, further study of induction mechanisms is needed. Preliminary analysis of SELDI-TOF-MS showed that there were a few differentially expressed proteins in the regenerated tissue extracts after liver injury, which are likely to be the molecular basis of their stem cell-inducing activity[20]. The results of reverse hybridization showed that the inducing directional differentiation of regenerated tissue extracts of liver injury on bone marrow mesenchymal stem cells acts mainly through regulation of the Wnt/β-catenin signal pathway. Many researchers have shown that abnormal activation of the Wnt/β-catenin signal pathway is closely related to tumorigenesis[21-24]. It can be inferred that differentiation-inducing effects of regenerated tissue extracts after liver injury on HCC may be related to the Wnt/β-catenin signaling pathway.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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References

[1] McGlynn KA, Tarone RE, El-Serag HB. A comparison of trends in the incidence of hepatocellular carcinoma and intrahepatic cholangiocarcinoma in the United States. *Cancer Epidemiol Biomarkers Prev* 2006; **15**(6): 1198–1203.

[2] Gocek E, Marcinkowska E. Differentiation therapy of acute myeloid leukemia. *Cancers (Basel)* 2011; **3**(2): 2402–2420.

[3] Huang ME, Ye YC, Chen SR, Chai JR, Lu JX, Zhoa L, et al. Use of all-trans retinoic acid in the treatment of acute promyelocytic leukemia. *Blood* 1988; **72**(2): 567–572.

[4] Adjiri A. Identifying and targeting the cause of cancer is needed to cure cancer. *Oncol Ther* 2016; **4**(1): 17–33.

[5] Schulenburg A, Blatt K, Cerny-Reiterer S, Sadovnik I, Herrmann H, Marian B, et al. Cancer stem cells in basic science and in translational oncology: can we translate into clinical application? *J Hematol Oncol* 2015; **8**:16.

[6] Conti L, Ruiu R, Barutello G, Macagno M, Bandini S, Cavallo F, et al. Microenvironment, oncoantigens, and antitumor vaccination: lessons learned from BALB-neuT mice. *Biomed Res Int* 2014; **2014**: 534969.

[7] Dragu DL, Necula LG, Bleotu C, Diaconu CC, Chivu-Economescu M. Therapies targeting cancer stem cells: current trends and future challenges. *World J Stem Cells* 2015; **7**(9): 1185–1201.

[8] Chen W, Dong J, Haiech J, Kilhoffer MC, Zeniou M. Cancer stem cell quiescence and plasticity as major challenges in cancer therapy. *Stem Cells Int* 2016; **2016**: 1740936.

[9] Reagan MR, Rosen CJ. Navigating the bone marrow niche: translational insights and cancer-driven dysfunction. *Nat Rev Rheumatol* 2016; **12**(3): 154–168.

[10] Lopez-Ayllon BD, Moncho-Amor V, Abarrategi A, De Cáceres II, Castro-Carpeño J, Belda-Iniesta C, et al. Cancer stem cells and cisplatin-resistant cells isolated from non-small-lung cancer cell lines constitute related cell populations. *Cancer Med* 2014; **3**(5): 1099–1111.

[11] Zhang H, Chang WJ, Li XY, Zhang N, Kong JJ, Wang YF. Liver cancer stem cells are selectively enriched by low-dose cisplatin. *Braz J Med Biol Res* 2014; **47**(6): 478–482.

[12] Castelli G, Pelosi E, Testa U. Liver cancer: molecular characterization, clonal evolution and cancer stem cells. *Cancers* 2017; **9**(9): 127.

[13] Wilson GS, Hu Z, Duan W, Tian A, Wang XM, McLeod D, et al. Efficacy of using cancer stem cell markers in isolating and characterizing liver cancer stem cells. *Stem Cells Dev* 2013; **22**(19): 2655–2664.

[14] Liebermann D, Sachs L. Co-regulation of type C RNA virus production and cell differentiation in myeloid leukemic cells. *Cell* 1978; **15**(3): 823–835.

[15] Breitman TR, Selonick SE, Collins SJ. Induction of differentiation of the human promyelocytic leukemia cell line (HL-60) by retinoic acid. *Proc Natl Acad Sci U S A* 1980; **77**(5): 2936–2940.

[16] Airoldi I, Meazza R, Croce M, Di Carlo E, Piazza T, Cocco C, et al. Low-dose interferon-gamma-producing human neuroblastoma cells show reduced proliferation and delayed tumorigenicity. *Br J Cancer* 2004; **90**(11): 2210–2218.

[17] Oehme I, Deubzer HE, Wegener D, Pickert D, Linke JP, Hero B, et al. Histone deacetylase 8 in neuroblastoma tumorigenesis. *Clin Cancer Res* 2009; **15**(1): 91–99.

[18] Asklund T, Appelskog IB, Ammerpohl O, Ekstrom TJ, Almqvist PM. Histone deacetylase inhibitor 4-phenylbutyrate modulates glial fibrillary acidic protein and connexin 43 expression, and enhances gap-junction communication, in human glioblastoma cells. *Eur J Cancer* 2004; **40**(7): 1073–1081.

[19] Liu X, Yang JM, Zhang SS, Liu XY, Liu DX. Induction of cell cycle arrest at G1 and S phases and cAMP-dependent differentiation in C6 glioma by low concentration of cycloheximide. *BMC Cancer* 2010; **10**: 684.

[20] Fan X, Hong H, Tang F, Liu F, Chen S, Zhao G. Comparision of expression profiles of serum proteins in the rat model of liver injury by using SELDI-TOF-MS technique. *Chin J Pathophysiol* 2007; **23**: 2476–2478.

[21] Novellasdemunt L, Antas P, Li VS. Targeting Wnt signaling in colorectal cancer. A review in the theme: cell signaling: proteins, pathways and mechanisms. *Am J Physiol Cell Physiol* 2015; **309**(8): C511–C521

[22] Xue J, Chen Y, Wu Y, Wang Z, Zhou A, Zhang S, et al. Tumour suppressor TRIM33 targets nuclear beta-catenin degradation. *Nat Commun* 2015; **6**: 6156.

[23] Mohammed MK, Shao C, Wang J, Wei Q, Wang X, Collier Z, et al. Wnt/beta-catenin signaling plays an ever-expanding role in stem cell self-renewal, tumorigenesis and cancer chemoresistance. *Genes Dis* 2016; **3**(1): 11–40.

[24] Monga SP. Beta-catenin signaling and roles in liver homeostasis, injury, and tumorigenesis. *Gastroenterology* 2015; **148**(7): 1294–1310.