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ORIGINAL ARTICLE

Tissue factor pathway inhibitor-2 induced hepatocellular carcinoma cell differentiation

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Abstract To investigate the effect of over-expression of tissue factor pathway inhibitor-2 (TFPI-2) on the differentiation of hepatocellular carcinoma (HCC) cells (Hep3B and HepG2). The TFPI-2 recombinant adenovirus (pAd-TFPI-2) was constructed using the pAdeasy-1 vector system. Transfected by pAd-TFPI-2, the cell proliferation of HCC cells was evaluated by CCK-8 assay, flow cytometry was used to detect cell apoptosis and CD133 expression. Real-time PCR and Western blot were used to detect the expression levels of markers of hepatocellular cancer stem cells (CSC) and hepatocytes. The over-expression of TFPI-2 significantly suppressed cell proliferation, induced apoptosis, and dramatically decreased the percentage of CD133 cells, which was considered as CSC in HCC. Real-time PCR and Western blot showed that the expression of markers of CSC in Hep3B cells and HepG2 cells infected with pAd-TFPI-2 was markedly lower than those of the control group ($P < 0.05$), while the expression of markers of hepatocytes was significantly increased ($P < 0.05$). Hence, TFPI-2 could induce the differentiation of hepatocellular carcinoma cells into hepatocytes, and is expected to serve as a novel way for the treatment of HCC.

1. Introduction

Hepatocellular carcinoma is one of the malignancies with a high incidence in the world, and its incidence is increasing (Forner et al., 2012; Kim and Park, 2014). Hepatocellular carcinoma has a silent onset with no symptoms in the early stage, while it progresses rapidly. Currently the treatment methods for hepatocellular carcinoma are mainly hepatic resection, hepatic transplantation, and drug therapy. However, only 10–20% of the patients with HCC can be surgically excised, yet attended with a high frequency of recurrence (Liu et al., 2014). Further, HCC is chemoresistant and the current drug therapy is associated with limited efficacy. The prognosis of HCC patients is generally poor (Altekruse et al., 2014). Although new strategies have been applied for HCC treatment, efficacies are still beyond satisfactory (Kozyreva et al., 2011). Therefore, it is of immense importance to seek some new ways for the treatment of hepatocellular carcinoma.

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Tissue factor pathway inhibitor-2 (TFPI-2) is also known as matrix-associated serine protease inhibitor (MSPI) and placental protein 5 (PP5) (Rao et al., 1995; Kisiel et al., 1994). TFPI-2 is a kunitz-type serine proteinase inhibitor, which is abundantly expressed in a variety of human tissues such as liver, pancreas, skeletal and directionally secreted into the extracellular matrix (ECM) (Miyagi et al., 1994; Sugiyama et al., 2002; Herman et al., 2001). TFPI-2 is thought to negatively regulate the enzymatic activity including matrix metalloproteinase (MMP), plasmin, cathepsin G, trypsin, and plasma kallikrein (Stamenkovic, 2003; Kempaiah et al., 2007). Previous studies have suggested that the expression of TFPI-2 is down-regulated in many malignant tumors, including breast cancer, gastric stromal tumor, cervical cancer, gliomas and non-small-cell lung cancer, and low expression of TFPI-2 was associated with poor prognosis in cancer patients (Wang et al., 2014; Zhang et al., 2013; Rao et al., 2001; Rollin et al., 2005; Xu et al., 2013).

The therapy of induced differentiation of tumors is proposed in recent years, which indicates a new direction for the treatment of hepatocellular carcinoma. Commonly used differentiation-inducing agents are mostly substances that might work on other malignancies. But generally speaking, the therapy of induced differentiation of hepatocellular carcinoma has not yet yielded satisfactory results. It has been reported that transmembrane protease, serine 4 (TMPRSS4) is upregulated by the silencing of TFPI-2 through aberrant DNA methylation in non-small-cell lung cancer (Hamamoto et al., 2015). TMPRSS4 has been shown to be an important regulator during the epithelial-mesenchymal transition (EMT) in human epithelial cancer cells (Li et al., 2011). EMT is a physiological mechanism which is present during development, including mesoderm formation and neural tube formation (Kalluri and Weinberg, 2009). Previous studies showed that the EMT process may facilitate the generation of cancer cells with the mesenchymal traits needed for dissemination as well as the self-renewal properties needed for initiating secondary tumors (Hollier et al., 2009). Our previous studies indicated that TFPI-2 could not only inhibit the proliferation, invasion and metastasis of Hep3B and HepG2, but also significantly reduce the expression and secretion of alpha-fetal protein (AFP), a maker of HCC (Xu et al., 2011). Therefore, we hypothesize that TFPI-2 may show an effect on inducing the differentiation of hepatocellular carcinoma cells (HCC) into mature hepatocytes and serve as a novel way for the treatment of hepatocellular carcinoma.

2. Materials and methods

2.1. Construction of adenoviral vectors

The sequence of TFPI-2 gene coding sequence was amplified by PCR. The shuttle plasmid and the TFPI-2 DNA fragment were bound using the T4DNA ligase (TaKaRa, Japan) after the restriction enzyme digestion. The sequence was identified via DNA sequencing and restriction enzyme digestion. The pAdtrack-cmv-TFPI-2 and the pAdEasy-1 were co-transformed into *Escherichia coli* BJ5183 with backbone vector AdEasy-1 for homologous recombination. The recombinant plasmid pAd-TFPI-2 digested by Pac I (Fermentas, USA) was used to transfect Hek293 cells (Cellbank, China) by Lipofectamine™ 2000 (Invitrogen, USA) for further packaging and amplification of the virus.

2.2. Cell culture and transfection

Hepatoma cell lines HepG2 and Hep3B were obtained from the American Type Culture Collection (ATCC, USA). The cells were grown in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (Gibico, USA), 1.0% glutamine, 100 μg/ml streptomycin and 100 μg/ml penicillin in a humidified atmosphere containing 5% CO_2 at 37 °C. The virus was added to the cell monolayers. Cells were then incubated for 2 h to complete the transfection of virus into the cells. The serum-free medium was replaced with serum-containing medium and cells were cultured for 48 h.

2.3. RT-PCR

Cells were harvested in Trizol (TaKaRa, Japan), and total RNA was isolated according to the manufacturer's instructions. After the RNA was reversely transcribed into cDNA, the change in the expression of TFPI-2 was detected using PCR. The cDNA was synthesized from 1 μg RNA as the template using RT-PCR kit (Takara, Japan). The original amount of TFPI-2 and β-actin was detected via PCR with Premix Taq (Takara, Japan). The primers were synthesized by The Beijing Genomics Institute (BGI, China) as follows: TFPI-2 sense 5′-ATAGGATCCACATGGACCCGCTCGC-3′ and antisense 5′-GGCCTCGAGAAATTGCTTCTTCCGAATTTCC-3′, β-actin sense 5′-GAGTCAACGGATTTGGTCGT-3′ and antisense 5′-GACAAGCTTCCCGTTCTCAG-3′. To study TFPI-2 gene expression, the PCR was initiated by a decontamination (95 °C for 5 min) and denaturation step (95 °C, 30 min), followed by 30 cycles at 60 °C for 30 s and at 72 °C for 40 s. The level of TFPI-2 mRNA was evaluated by the ratio of density of TFPI-2 to β-actin.

2.4. Western blot

The cells were collected at 72 h after infection. The cultures were washed several times with phosphate-buffered saline (PBS). Total proteins were harvested in cell lysates supplemented with PMSF (1 mmol/l) to inhibit the proteases. The samples were boiled for 5 min and the proteins were separated by sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) on 12% polyacrylamide gels. After electrophoresis, proteins were transferred onto nitrocellulose membranes and blocked with 5% non-fat milk for 2 h at 37 °C. After blocking, the membranes were incubated for 12 h at 4 °C with anti TFPI-2 antibody (Santa Cruz, USA) diluted by TBST. After several washes, the membranes were incubated horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG secondary antibody (Santa Cruz, USA). After washing, the blots were detected by Odyssey Infrared Imaging System (LI-COR).

2.5. Flow cytometry analysis

Flow cytometry was used to detect the cell apoptosis and CD133 expression. Briefly, cells (3×10^5/well) were seeded in a six-well plate, and infected with adenovirus. After 72 h, cells

were harvested by trypsinization and suspended in PBS. The level of apoptosis of cells was detected with Annexin V-PE Apoptosis Detection Kit (Affymetrix, USA). To detect CD133 expression in hepatoma cells, PE-conjugated CD133/1 (Miltenyi, GER) was used as primary antibody. Isotype-matched mouse immunoglobulin served as controls. The cell suspension was analyzed with a FACS Caliber flow cytometer using CellQuest software (Becton, CA).

2.6. Cell proliferation assay

To test the inhibitory effect of TFPI-2 on human hepatoma cell proliferation, Hep3B and HepG2 cells (3×10^3/well) were seeded in a 96-well plate, respectively, and cultured for 12 h. The cells were then infected with adenovirus as described above. Every 24 h, the cells were harvested and 100 μl cell suspension was added to each well in 96-well plates for a total of 5 days. 10 μl of the CCK-8 solution (Sigma, CA) was added to each well of the plate and incubated for 4 h at 37 °C. The number of metabolically active mitochondria and viable cells was measured colorimetrically at 450 nm. Each experiment was repeated at least three times with each treatment given in triplicate.

2.7. Detection of CSC markers and hepatocyte markers

Primers for these transcripts were listed in Supporting Table 1. cDNA was synthesized with an oligo (dT) primer and M-MLV reverse transcriptase according to a standard protocol. The original amount of the specific transcripts was detected via real-time PCR with a SYBR PCR Kit (TaKaRa, Japan). The expression of specific transcripts was normalized against that of β-actin. Western blot analysis of c-Myc and β-catenin was performed according to the manufacturer's instructions (Santa Cruz, USA). After incubating with primary and secondary antibodies, the blots were detected by LI-COR.

2.8. Statistical treatment

The software SPSS 17.0 was adopted for the analysis of various data, which were represented as mean ± standard deviation ($\overline{x} \pm s$) for triplicate samples. Student's *t*-test was used to compare the difference between groups. A P value of <0.05 was considered to be statistically significant.

3. Results

3.1. Identification of the recombinant adenovirus

Hep3B cells infected with recombinant adenovirus were cultured for 48 h, green fluorescent protein (GFP) reached over 80%, indicating that the recombinant adenovirus could efficiently infect Hep3B cells in vitro (Fig. 1A). After Ad-TFPI-2 infected Hep3B cells for 48 h, the expression level of TFPI-2 mRNA in Hep3B cells was detected by PCR. Quantitative evaluation after Ad-TFPI-2 infection was normalized to that of β-actin and revealed that increased expression of TFPI-2 mRNA was found in the Hep3B-TFPI-2 cells compared with Hep3B-GFP ($P < 0.01$) (Fig. 1B). Expression of TFPI-2 protein in Hep3B-TFPI-2 cells, Hep3B-GFP and Hep3B cells, analyzed by Western blot with anti TFPI-2 antibody, was shown in Fig. 1C. Western blot analysis for TFPI-2 protein expression in three groups indicated TFPI-2 protein expression was increased in Hep3B-TFPI-2 cells, as compared with those in the control groups. We detected 3 different glycosylated isoforms of TFPI-2, and all 3 isoforms were upregulated by Ad-TFPI-2 infection in Hep3B cells. These results showed that TFPI-2 was successfully transfected into Hep3B cells.

3.2. TFPI-2 induces apoptosis in Hep3B cells

To determine the effect of TFPI-2 on cell apoptosis in HCC, flow cytometry was performed to detect the population of apoptotic cells in Hep3B cells. It could be observed from Fig. 2A that up to (12.53 ± 0.36)% of Hep3B cells became apoptotic after TFPI-2 gene delivery, while in the GFP-infected group and un-infected group the apoptosis rate was (2.72 ± 0.18)% and (2.33 ± 0.19)%, respectively. The difference of cell apoptosis rate in three groups had obvious statistical significance ($P < 0.05$). These results have suggested that TFPI-2 could induce the apoptosis of Hep3B cells.

3.3. TFPI-2 inhibits proliferation of Hep3B and HepG2 cells

As shown in Fig. 2B, the cell proliferation of Hep3B-TFPI-2 cells suppressed significantly from day 3, as compared with those in 2 control groups ($P < 0.05$). There was no significant difference between Hep3B-GFP cells and Hep3B cells ($P > 0.05$). Cell proliferation in the HepG2-TFPI-2 group was also significantly inhibited from day 4 ($P < 0.05$), but not inhibited in the two control groups ($P > 0.05$).

3.4. TFPI-2 increases the expression of hepatocyte cell markers

The effect of TFPI-2 on the hepatocyte cell markers of Hep3B and HepG2 cells was detected by real-time PCR. As shown in Fig. 3, the over-expressed TFPI-2 could increase the expression of hepatocyte markers, including 4-hydroxyphenylpyruvate dioxygenase (HPD), cytochrome P4501α2 (CYP1α2), biliverdin reductase (BR), phosphoenolpyruvate carboxykinase (PEPCK), glucose-6-phosphatase (G-6-P), aldolase B (ALDOB), glycogen synthetase 2 (GYS2) and glutamine synthetase (GS). The results have suggested that TFPI-2 exhibits effects on inducing the differentiation of hepatocellular carcinoma cells into mature hepatic cells.

3.5. TFPI-2 reduces the expression of CSC markers

CD133 is considered to serve as targets for identifying cancer stem cells in HCC. As shown in Fig. 4A, flow cytometry showed that $CD133^+$ cells accounted for (57.69 ± 2.23)% of Hep3B cells infected with Ad-TFPI-2, significantly less than that of Hep3B cells infected with GFP [(88.46 ± 1.25)%, $P < 0.01$]. The percentage of $CD133^+$ cells in HepG2 decreased from (57.39 ± 1.17)% to (41.64 ± 1.82)% after infection with TFPI-2 ($P < 0.05$). Similarly, compared with the control group, the expression of CD133 mRNA was significantly down-regulated in Hep3B cells and HepG2 cells infected with Ad-TFPI-2. Furthermore, the expression of c-Myc and β-catenin mRNA was down-regulated, which are

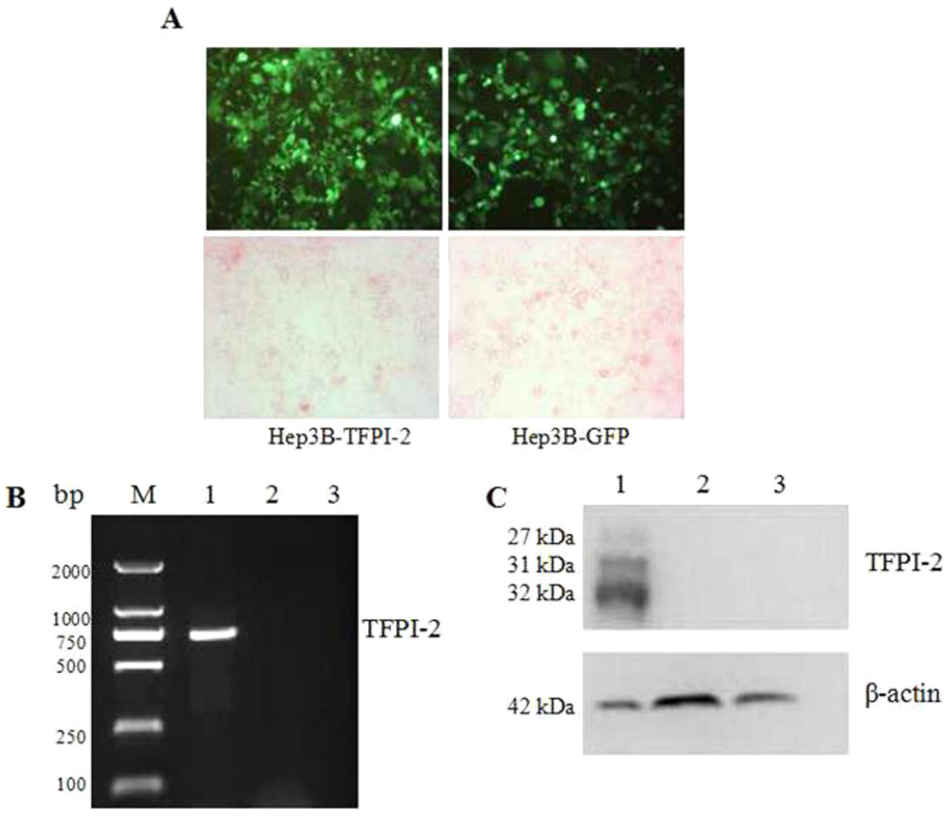

Figure 1 Identification of TFPI-2 mRNA and protein expression in Hep3B cells. A, 48 h after AdTFPI-2 and Ad-GFP infection, the infection rates of Hep3B could reach more than 80% in vitro indicated by green fluorescent protein (100 ×). B, The expression of TFPI-2 mRNA in Hep3B was detected by PCR. The high expression of TFPI-2 mRNA was observed in Hep3B-TFPI-2 cells. There was no expression of TFPI-2 mRNA in Hep3B-GFP or Hep3B cells. Lane 1, DNA marker DL 2000; Lane 2, Hep3B-TFPI-2 cells; Lanes 3, Hep3B-GFP cells; Lanes 4, Hep3B-GFP cells. C. After infection with AdTFPI-2 and Ad-GFP for 72 h in Hep3B, the total proteins were extracted and analyzed for the expression of TFPI-2 protein with Western blot. Three different glycosylation isoforms of TFPI-2 were detected in Hep3B-TFPI-2 cells. There was no expression of TFPI-2 protein in Hep3B-GFP or Hep3B cells. Lane 1, Hep3B-TFPI-2 cells; Lanes 2, Hep3B-GFP cells; Lanes 3, Hep3B cells.

highly expressed in embryonic stem cells and involved in the maintenance of pluripotency (Fig. 4B). Western blot results also showed that the expression of c-Myc and β-catenin proteins was reduced in Hep3B cells and HepG2 cells infected with Ad-TFPI-2 (Fig. 4C).

4. Discussion

TFPI-2, also known as placental protein (PP5), is identified as a tumor suppressor gene (Bretz et al., 2012). As a member of the Kunitz structure superfamily, TFPI-2 is a broad-spectrum inhibitor of serine protease. Since the promoter of TFPI-2 is rich in CPG islands, its expression is silenced in many malignant tumors through epigenetic modifications, including promoter methylation and histone deacetylation (Dong et al., 2015; Glockner et al., 2009). In addition, the aberrant splicing form of TFPI-2 was detected during cancer progression, which represented an untranslated form providing another mechanism (Bretz et al., 2012). Moreover, TFPI-2 could mediate dephosphorylation of residues outside the T-loop of ERK, which may directly impact kinase function (Mazalouskas et al., 2014). The ERK1/2 pathway integrates various cytosolic signals to regulate cellular proliferation, differentiation, and apoptosis, which contributes to the formation and development of a variety of tumors (Deng et al., 2013; George et al., 2007).

Tumors are organized in a hierarchy of heterogeneous cell populations with different biologic properties comprising proliferating transit-amplifying cells, terminally differentiated cells, and dying cells and that the populations consist of cancer stem cells (CSCs). Some of the proliferating cells do not differentiate into mature cells, which could continue to proliferate. The CSCs are thought to maintain tumor cells self-renewal capacity, high proliferation rate and are more resistant to chemotherapy than differentiated cancer cells (Ciurea et al., 2014; Puglisi et al., 2013). Differentiation therapy could force hepatocellular carcinoma cells to differentiate and lose self-renewal capacity. Cell differentiation is assumed to be regulated by an informational network, including transacting factors, soluble transmitters and cell-matrix adhesion molecules. But, to our knowledge, little is known on the role of TFPI-2 inducing differentiation in hepatocellular carcinoma.

To study the relationship between TFPI-2 and tumor cells differentiation, we constructed the recombinant adenovirus Ad-TFPI-2 to enable TFPI-2 to overexpress in hepatocellular carcinoma cells, and proved that Ad-TFPI-2 could infect

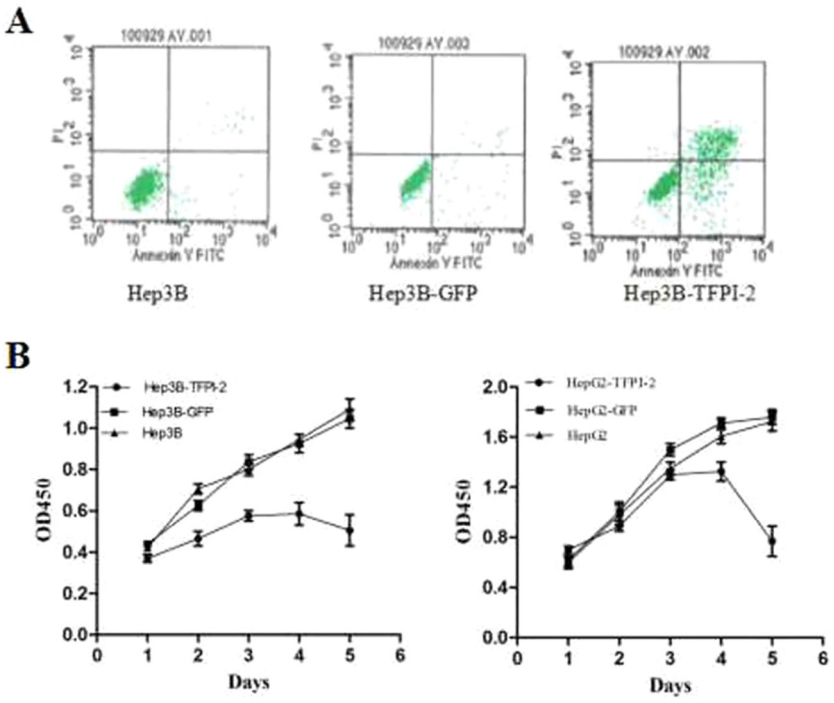

Figure 2 A, Detection of the effect of TFPI-2 on the apoptosis of Hep3B cells using flow cytometry. Hep3B cells (3×10^5/well) were seeded in a six-well plate. After 72 h infection with adenovirus, apoptosis cells were detected by flow cytometry. Each treatment was performed in triplicate, and the assays were repeated at least 3 times. B, TFPI-2 suppresses hepatoma cell proliferation. The suppression rate was significant on the third day (Hep3B) and the fourth day (HepG2), respectively, after TFPI-2 infection compared with 2 control groups. $^{*}P < 0.05$ *vs* 2 control groups.

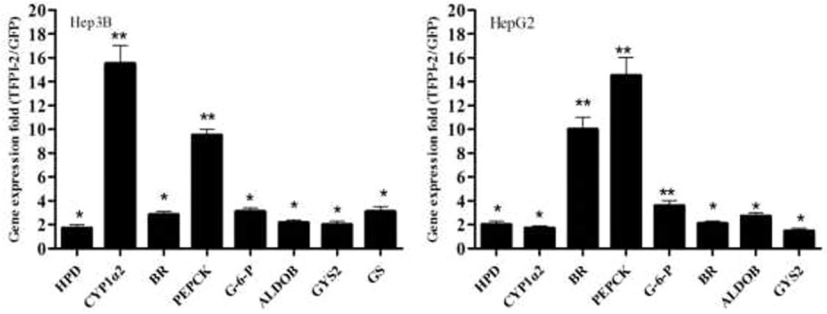

Figure 3 Gene expression folds of the Ad-TFPI-2-infected group versus the Ad-GFP-infected group. TFPI-2 up-regulates expression of hepatocyte markers in Hep3B cells and HepG2 cells. The expression of characteristic hepatocyte markers was detected by real-time PCR, and the mRNA expression levels were normalized against β-actin. Significant increases in expression of hepatocyte markers were detected in Hep3B cells and HepG2 cells infected by Ad-TFPI-2 including 4-hydroxyphenylpyruvate dioxygenase (HPD), cytochrome P4501α2 (CYP1α2), biliverdin reductase (BR), phosphoenolpyruvate carboxykinase (PEPCK), glucose-6-phosphatase (G-6-P), aldolase B (ALDOB), glycogen synthetase 2 (GYS2) and glutamine synthetase (GS). $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* control group.

Hep3B cells efficiently and overexpress TFPI-2. The expression levels of hepatocyte makers, CD133, c-Myc and β-catenin were analyzed. Our data suggested that TFPI-2 induced apoptosis and inhibited proliferation of HCC. Moreover, the over-expression of TFPI-2 could elevate the expression of hepatocyte makers and reduce the expression of stemness gene.

We chose Hep3B and HepG2 for the present study because they are certified to contain a small proportion of CSCs, and the expression of TFPI-2 was silenced, which were able to overexpress the TFPI-2 protein and mRNA by infecting Ad-TFPI-2. Our results demonstrated that stable expression of TFPI-2 inhibited the cell proliferation rate 2 times in Hep3B-TFPI-2 cells. Our findings are consistent with previous reports asserting that the over-expression of TFPI-2 induced the apoptosis and inhibited proliferation of cancer cells, such as hepatocellular carcinoma (Wong et al., 2007), meningioma (Kondraganti et al., 2006) and esophageal carcinoma (Ran et al., 2009). TFPI-2 could regulate tumor angiogenesis by reducing synthesis of the VEGF receptor and affect the expression of several genes involved in oncogenesis, invasion and

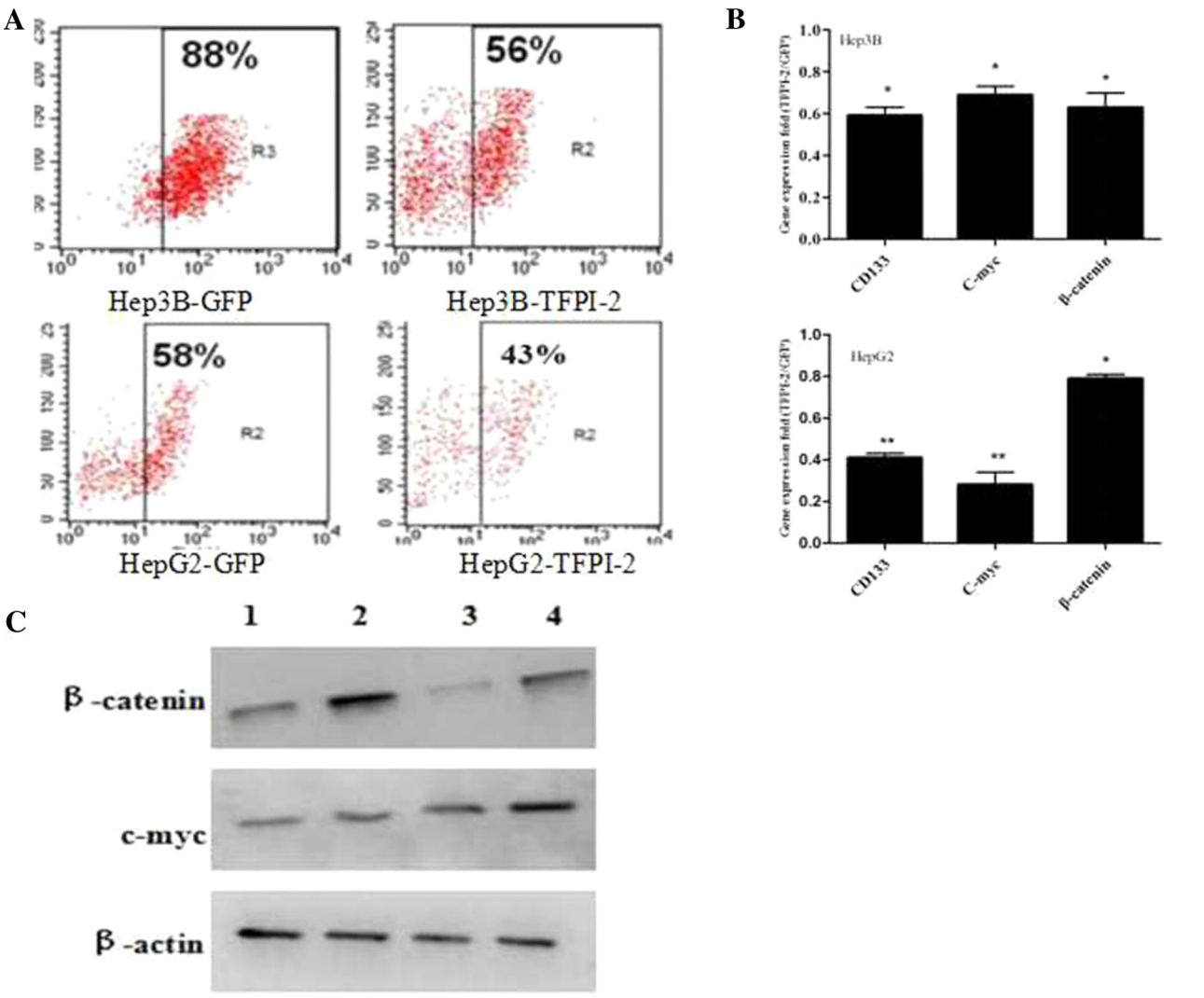

Figure 4 TFPI-2 reduced the stemness gene expression in Hep3B cells and HepG2 cells. A, Flow cytometry analysis confirmed reduced proportion of $CD133^+$ cells in Hep3B and HepG2 after infection with Ad-TFPI-2 (Hep3B, $P < 0.01$; HepG2, $P < 0.05$). B, Moreover, CD133, c-Myc and β-catenin mRNA expression was also reduced compared with the control groups. mRNA expression folds of the TFPI-2 group versus the GFP group were shown and normalized against β-actin. C, Western blot analysis for the expression of c-Myc and β-catenin in Ad-TFPI-2 infected Hep3B cells and HepG2 cells. Reduced expression of c-Myc and β-catenin proteins was observed in both Hep3B and HepG2. Lane 1, Hep3B-TFPI-2 cells; Lane 2, Hep3B-GFP cells; Lanes 3, HepG2-TFPI-2 cells; Lanes 4, HepG2-GFP cells.

apoptosis (Chand et al., 2004). In addition, TFPI-2 could also activate caspase-mediated, pro-apoptotic signaling pathways and induces apoptosis (George et al., 2007). Of course, further studies are required to investigate whether these mechanisms are also regulated within HCC cells by TFPI-2.

Recent studies suggest that malignant tumors can be viewed as an abnormal organ, which CSCs have escaped the normal limits of self-renewal, giving rise to abnormally differentiated cancer cells that contribute to tumor progression. Targeting CSCs by inducing differentiation is an encouraging way of therapy for HCC. CD133, also called Prominin-1, is a product of a single-copy gene on chromosome 4 (4p15.33) in human. Liver cancer stem cells, whose specific marker was CD133, were isolated from liver cancer cell lines, and it was found that these cells accounted for only a very small proportion of the cells, while they had strong capacity in colony formation and tumorigenesis (Ma et al., 2007). CD133, as a specific surface molecule for sorting cancer stem cells, has been identified in colon cancer (Maria et al., 2013), hepatocellular carcinoma (Mishra et al., 2009) and lung cancer (Kristen et al., 2014). As one of specific markers of cancer stem cells, CD133 holds great significance in the development and progression of HCC. Our results revealed that the percentage of $CD133^+$ cells was significantly decreased in both Hep3B and HepG2 cells infected with AdTFPI-2. It was reported that the expression of TFPI-2 was inversely correlated with the expression of CD133 (Chu et al., 2015). Moreover, the expression of c-Myc and β-catenin was also demonstrated to be down-regulated by TFPI-2 in Hep3B and HepG2. β-catenin is one of the key effectors responsible for transduction of the signal to the nucleus and it triggers transcription of Wnt-specific genes responsible for the control of cell fate decisions. EMT plays

an important role during embryonic development and drives cancer cells invasion and metastases (Thiery et al., 2009). In transitions related to cancer progression, activating mutations of β-catenin could enhance transcription, which might have consequences for EMT progression. TFPI-2 could up-regulate the expression of TMPRSS4, which plays an important regulator during the epithelial-mesenchymal transition (EMT) (Li et al., 2011). In the nucleus, β-catenin activates transcription of Wnt/β-catenin-target genes such as c-myc, which is involved in cell proliferation as well as differentiation. Consistently, our results illustrated a list of elevated genes that are markers for hepatocyte maturation.

5. Conclusions

We successfully constructed the recombinant adenovirus Ad-TFPI-2 that could effectively infect HCC cells and express TFPI-2. The overexpression of TFPI-2 can inhibit the proliferation and induce the apoptosis of HCC cells, and increase the expressions of markers of hepatic cells while decrease those of makers of stem cells, which indicates TFPI-2 can induce the differentiation of hepatocellular carcinoma cells into mature hepatic cells. However, its mechanism remains elusive and requires further studies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.sjbs.2016.09.003.

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